STUDIES ON THE HYALURONIDASE ENZYME PURIFIED FROM THE VENOM OF CHINESE RED SCORPION BUTHUS MARTENSI KARSCH

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NATIONAL UNIVERSITY OF SINGAPORE
2010

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A thesis submitted by

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(B.Med., M.Med.)

for the degree of

DOCTOR OF PHILOSOPHY
in the
NATIONAL UNIVERSITY OF SINGAPORE

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National University of Singapore
2010

Acknowledgements

I would like to take this opportunity to express my sincere appreciation to my supervisor **Prof. P. Gopalakrishnakone**, Department of Anatomy, National University of Singapore. During my study in Anatomy, I owed much to his great patience, academic guidance and endlessly encouragement. It is my luck to study under his supervision.

I'd also like to thank **Prof. Bay Boon Huat**, the head of Department of Anatomy, National University of Singapore, for his management to make the whole department as a big family and hence I could enjoy the stay in the department.

I will not forget the great support from **Dr. Gao Rong**. I have learned much from him, from the understanding of the science to the techniques of the experiments. I felt so happy to meet such a big brother in the lab.

I would also thank **Dr. M.M.Thwin**, the senior member of Venom and Toxin Research Programme, who helped me in writing the manuscript, patiently listened to my queries and unselfishly shared his experience. He was always available when I needed the help.

I highly appreciate the kindly help of **Mr. Meng Jun**, **Dr. R. Saminathan**, **Ms. Hema D/O Jethanand** for my research activities and thank as well to **Dr. P. Saravanan, Dr. A. Pachiappan, Dr. Perumal Samy**, and all other Venom and Toxin Research Programme members, for maintaining a favorable working environment.

I would like to thank **Ms Yong Eng Siang, Ms Ng Geok Lan**, for their efficient organization to keep the lab clean and safe, **Ms Violet Teo**, **Ms Carolyne Ang** and **Ms Diljit Kour d/o Bachan Singh**, for their secretarial assistance. I also wish to thank all the Department staffs and students; I will never forget the life in the Department.

I would also like to show the gratitude to my family. My parents' support and tolerance is always the drive for me to step forward.

Last but not least, I would acknowledge the National University of Singapore, for generously offering me a scholarship to complete this research work.

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Summary

The present work includes 1) screening of the biological activities in scorpion *Buthus martensi* Karsch (*Bm*K) crude venom; 2) the purification and characterization of the hyaluronidase enzyme (BmHYA1) from the venom of *Bm*K; 3) the cDNA cloning and expression of BmHYA1 and 4) the preliminary pharmacological study of BmHYA1.

Scorpion venom is a rich source for short neurotoxic peptides but this study indicates it also contains various high molecular weight (M.W.) proteins. A number of enzymatic activities have been detected in the present work including L-amino acid oxidase (LAAO), serine protease, and hyaluronidase. It is also possible to contain Phospholipase A_2 (PLA_2) and metalloproteinase. This work should be the pioneer in comprehensive investigation of the enzymatic proteins in scorpion *Bm*K venom.

The hyaluoridase from the crude venom of *Bm*K, later named as BmHAY1, was studied in detail. The enzyme was purified from the crude venom by a successive chromatography of gel filtration, ion-exchange and reversed-phase high-performance liquid chromatography (RP-HPLC). The homogeneity was manifested by dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS) and Edman degradation. MALDI-TOF result also showed its molecular weight of 48,696 Da. Its N-terminal amino acids were determined by Edman degradation and showed homologies to other venom hyaluronidases to some degree. BmHYA1 has an optimal temperature of 50 ^{o}C and

optimal pH of 4.5. Its K_m and V_{max} are determined to be 95.3 μg/mL and 3.9 μg/min, respectively. Additionally, the enzyme can hydrolyze the substrate hyaluronan into tetrasacchrides.

Rapid amplification of cDNA ends- polymerase chain reaction (RACE PCR) technique was used to clone the 3' end BmHYA1 cDNA sequence. The 5' degenerate primer was designed based on known N-terminal sequence hence the whole mature BmHYA1 sequence was deduced. This is also the first hyaluronidase full protein sequence from the scorpion species. The alignment shows it has some homologies (up to 34%) to other Glycol-Hydro-56 family members. The phylogenetic analysis indicates early divergence and independent evolution of BmHYA1 from other hyaluronidase family members. The recombinant BmHYA1 was expressed in *E.coli* but did not show the activity.

The treatment with BmHYA1 to MDA-MB-231 breast cancer cells gave rise to the removal of hyaluronan from the cell surfaces. The further study about its effect on CD44 molecules showed that the environmental hyaluronidase (BmHYA1) can modulate the expression of CD44 variant 6.

Publications

Peer Reviewed Papers:

1. **Feng, L.**, Gao, R., Gopalakrishnakone, P., (2008) Isolation and characterization of a hyaluronidase from the venom of Chinese red scorpion *Buthus martensi*. *Comp Biochem Physiol C Toxicol Pharmacol*. **148**:250-7.

2. **Feng, L.**, Gao, R., Meng, J., Gopalakrishnakone, P., Cloning and molecular characterization of BmHYA1, a novel hyaluronidase from the venom of Chinese red scorpion *Buthus martensi* Karsch. *Toxicon* (*In press*) doi: 10.1016/j.toxicon.2010.04.009

3. Saminathan, R., Pachiappan, A., **Feng, L.**, Rowan, E.G., Gopalakrishnakone, P., (2009) Transcriptome profiling of neuronal model cell PC12 from rat pheochromocytoma *Cellular and Molecular Neurobiology* **29**:533-48.

Conference Abstracts:

1. **Feng, L.**, Gao, R., Gopalakrishnakone, P., Characterization and biological activity study of a novel hyaluronidase from the venom of Asian scorpion *Buthus martensi* Karsch. 8th IST Asia-Pacific Congress on Animal, Plant and Microbial Toxins, Vietnam, 2008

2. Gao, R., **Feng, L.**, Gopalakrishnakone, P., A novel serine protease isolated from the venom of Asian scorpion *Buthus martensi* Karsch. 8th IST Asia-Pacific Congress on Animal, Plant and Microbial Toxins, Vietnam, 2008

3. **Feng L.**, Gao R., Gopalakrishnakone P. Cloning and molecular characterization of a novel serine protease, BMK-CBP, from the venom of Chinese red scorpion *Buthus martensi* Karsch. International Anatomical Sciences and Cell Biology Conference (IASCBC), Singapore, 2010

Abbreviations

AP	adapter primer
AUAP	abridged universal amplification primer
*Bm*K	*Buthus martensi* Karsch
BCV	*Bm*K crude venom
BVHYA	bee venom hyaluronidase
BPH-20	bovine PH-20
cDNA	complementary DNA
Da	dalton
DAB	3,3'-diaminobenzidine
DMEM	Dulbecco's Modified Eagle's Medium
DNA	deoxy ribonucleic acid
dNTP	deoxy nucleotide triphosphate
ECM	extracellular matrix
EDTA	ethylenediamine tetraacetic acid
FBS	fetal bovine serum
GSP	gene specific primer
GST tag	glutathione S-transferase tag
HPLC	high performance liquid chromatography
Human HYAL-1~4	human hyaluronidase-1~4
IPTG	isopropyl β-D-1-thiogalactopyranoside
kDa	kilodalton

LAAO	L-Amino Acid Oxidase
LB	lysogeny broth
MALDI-TOF	matrix assisted laser desorption ionisation time-of-flight
MS	mass spectrometry
mRNA	messenger ribonucleic acid
M.W.	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PBS	phosphate buffered saline
PLA_2	phospholipase A_2
RACR PCR	rapid amplification of cDNA ends- polymerase chain reaction
RBC	red blood cell
RNA	ribonucleic acid
Rpm	revolutions per minute
RT-PCR	reverse transcription polymerase chain reaction
PR-HPLC	reversed-phase high-performance liquid chromatography
S-2238	Bz-Ile-Glu-Gly-Arg-*p*Na
SDS-PAGE	dodecyl sulfate-polyacrylamide gel electrophoresis
SOC	super optimal broth
ss cDNA	single-stranded cDNA
TFA	trifluoroacetic acid
TIM	triose phosphate isomerase

TLC	thin layer chromatography
UAP	universal amplification primer
VGSCs	voltage-gated sodium channels

CHAPTER I

INTRODUCTION

1.1 Venomous animals and their venoms

Venomous animals produce lethal secretion known as venom from specialized venom glands. In animal kingdom, there are many creatures, e.g., snakes, scorpions, spiders and bees, etc., which may be different in morphology, habit, species and size, but they all share one remarkable specialty of producing the venoms.

Venomous animals employ venoms for defensive or offensive purpose. The venom can paralyze or even kill the victim in a very short time. The components in animal venoms accounting for these biological effects are mainly proteins or peptides. According to their molecular sizes, the venom proteins can be roughly classified into two groups: high M.W. enzymes, which are involved in various biochemical processes, and low M.W. peptides, which act mainly on numerous ion channels/receptors.

These properties make animal venoms a rich source for biomedical scientists in search of novel molecules to study biological phenomena or treat human disorders.

1.2 Scorpion biology

The scorpion is one of the most important sources of venom which has been widely studied. They are one of the oldest creatures which have been in existence on earth for millions of years since the middle Silurian (about 425~450 million years ago) period. Scorpions are widely distributed, with over 1,500 species reported so far

(Polis, 1990), and can be found in all the continents except Antarctica (Fig. 1.1). Scorpions belong to arthropods and have a large family. Under the class *Arachnida*, there are nine families: *Bothriuridae, Buthidae, Chactidae, Chaerilidae, Diplocentridae, Ischnuridae, Iuridae, Scorpionidae* and *Vaejovidae* (Sissom, 1990). The family *Buthidae*, containing 48 genera and more than 500 species, is supposed to be the largest and most widespread species among these families (Sissom, 1990). *Buthidae* is also considered as the medically important scorpion family (Fet and Lowe, 2000; Simard and Watt, 1990). Scorpion *Buthus martensi* Karsch (*Bm*K), the Chinese red scorpion (also called East Asian scorpion, note: scorpion *Mesobuthus tamales* is usually called Indian red scorpion), which belongs to *Buthidae* family, is the most commonly found scorpion in mainland China.

The *Bm*K Scorpion is yellowish to brown in color, and with the length (including the tail) of up to 6 cm, it is generally small in size as compared to other scorpion species (Fig. 1.2). It is not aggressive, and its venom toxicity is considered to be moderate and non-lethal to human (Goudet et al., 2002). The venom is produced and secreted from the venom gland which is located in the telson (the last segment of the metasoma. Fig. 1.2). In telson, there is a pair of venom glands each on either side of the middle septum (Fig. 1.3).

Fig. 1.1. Geographic distribution of scorpions whose venoms have been mostly studied. Aah, *Androctonus australis hector*; Amm, *Androctonus mauretanicus mauretanicus*; Be, *Buthus epeus*; Bom, *Buthus occitanus mardochei*; Bot, *Buthus occitanus tunetanus*; Ce, *Centruroides sculpturatus*; Clt, *Centruroides limpidus*; Css, *Centruroides suffuses suffuses*; Cn, *Centruroides noxius*; Lqq, *Leirus quinquestriatus quinquestriatus*; Lqh, *Leiurus quinquestriatus hebraeus*; *Bm*K, *Buthus martensi* Karsch; Bt, *Buthus tamulus*; Ts, *Tityus serrulatus*. (After Loret and Hammock, 2001)

Fig. 1.2. *Buthus martensi* Karsch (Chinese red scorpion). Wild-specimen from Xuzhou, Jiangsu Province, PR China. Inset: Segments of metasoma and telson (venom gland inside).

The medical significance of *Bm*K scorpion itself has been recorded for more than a thousand years. In China, during the Song Dynasty (A.D. 960-1279), the medical use of the *Bm*K scorpion body was recorded in the official pharmaceutical book *Kai Bao Ben Cao* (*Kai Bao*, referring to a time period from 968-975; *Ben Cao* means "herbs"). In another pharmacopoeia, *Ben Cao Gang Mu* (*Compendium of Materia Medica*, A.D.1578), which is probably the most famous Chinese pharmacopoeia book, the medicinal use of scorpions was described in detail as anti-epilepsy, analgesic, anticoagulant and anti-rheumatism agents.

1.3 Scorpion venom

Scorpion venom is produced and secreted by the venom glands. When needed, the scorpion erects the tail and stings the victim with its telson to inject the venom into the victim's body. The venom can also be milked by electrical stimulation. Generally, 1 gram of dry crude venom could be collected from 3000 scorpions (information from the venom supplier). The first drop of the venom (called pre-venom) is transparent and clear, but becomes milk-white and mucous later on. The components of the pre-venom are different from the mature venom (Inceoglu et al., 2003).

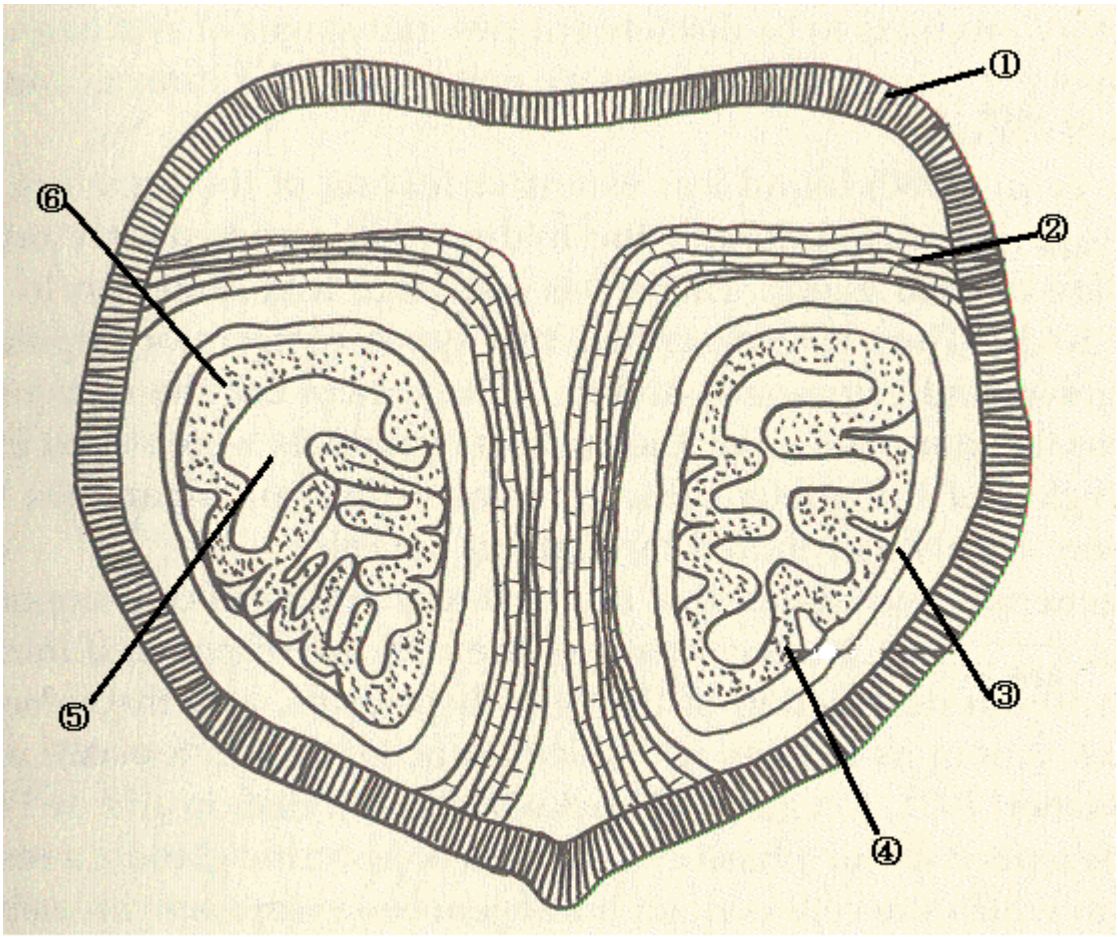


Fig. 1.3. Representative diagram of scorpion venom glands. ① cuticle ② skeletal muscle ③ capsule ④ venom gland ⑤ lumen ⑥ scretory cells. (After Snodgrass, 1952)

The venom of scorpion is a very complex mixture, which is composed of mucus, salts, various small neurotransmitters (e.g., serotonin, histamine, acetylcholine and norepinephrine), low M.W. peptides (mainly 3~8 kDa neurotoxins) and high M.W. enzymes (Polis, 1990; Martin-Eauclaire and Couraud, 1995; Nirthanan et al., 2002). The proteins in the venoms attract the most attention due to their significant medical/scientific applications.

Small peptides in scorpion venoms are mainly neurotoxins which may modulate various ion channels on excitable cells. Possessing a broad spectrum of specificity for ion channels, they are also valuable tools as molecular probes for the basic neuroscience research. Scorpion neurotoxins can be classified according to the different ion channels they target, though there are several other species of peptides that do not target ion channels.

1.3.1 Sodium channel toxins

The sodium channel toxin was the first neurotoxin purified from the scorpion venom (Rochat et al., 1967). They are usually long chain peptides (60~70 amino acids) with four disulfide bonds (Possani et al., 1999). On the basis of different targeted sites on sodium channel, they are further divided into α- and β-toxins (Couraud et al., 1982). Voltage-gated sodium channels (VGSCs) consist of an α subunit and two β subunits (β1 and β2). Scorpion α-toxins interact with the domain in α subunit (neurotoxin receptor site 3 of VGSCs), while β-toxins bind to the domain located in

β1 subunit (neurotoxin receptor site 4 of VGSCs) (Jover et al., 1988; Martin-Eauclaire et al., 1995). Hence, there is no competitive relationship between these two groups. The α-toxins can prolong the action potential, and therefore inhibit or slow down the inactive process of sodium channel. The β-toxins may increase sodium current by shifting the threshold of activation to hyperpolarized potentials (Couraud et al., 1982; Marcotte et al., 1997). The sodium channel scorpion toxins that specifically act on mammals and insects are classified into vertebrate sodium channel toxins and insect sodium channel toxins, respectively. The latter can be further divided into two groups: the excitatory and the depressant insect toxins. (Goudet et al., 2002; Martin-Eauclaire et al.,1995).

1.3.2 Potassium channel toxins

Potassium channels have a large family. Voltage-gated potassium channel blockers may inhibit cellular proliferation and suppress cellular activation, through the modulation of calcium influx (Wulff et al., 2009). Scorpion toxins can specifically target at three potassium channel types: the delayed rectifier potassium channels, the transient (or A-type) potassium channels and the calcium-dependent potassium channels (Hille, 1991). Based on structural homology, they fall into 17 sub-families, termed α-KTx_{1-17} (Tytgat et al., 1999; Zhang et al., 2004a; Wang et al., 2005). Most scorpion potassium channel blockers are relatively short peptides (30-40 amino acids) with three or four disulfide bridges (Garcia et al., 2001). On the other hand, some long chain potassium toxins have been purified or cloned from scorpion venoms and may

therefore form new classes (Yao et al., 2005; Legros et al., 1998). The scorpion potassium toxins have played a crucial role in elucidating the structure of KcsA channel, a potassium selective channel from *Streptomyces lividans* (Lu and MacKinnon, 1997; Garcia et al., 2001).

1.3.3 Calcium channel toxins

Voltage-gated calcium channels are involved in several physiological or pathological progresses, e.g., in pain pathways (Zamponi et al., 2009). Calcium channel toxins are able to exert pharmacological effect on these progresses and are considered to be the potential treatment of chronic pain (Norton and McDonough, 2008). Several calcium channel-related toxins were purified or cloned from scorpion venoms. Toxin II.6 from the venom of scorpion *Centruroides limpidus* showed inhibitory effect on the classical Ca^{2+} current activated at high membrane potentials. It was also reported to have a modulatory effect on sodium channel activities. (Alagón et al., 1988). A β scorpion toxin Tityus gamma, from the venom of scorpion *Tityus serrulatus,* was able to release calcium from the intracellular IP_3-sensitive calcium stores. (Fernandes et al., 2004). From the venom of scorpion *Pandinus imperator*, toxins called Tetrapandins were found to specifically inhibit the store-operated calcium entry in human embryonic kidney-293 cells, and were thought to be a new toxin class (Shalabi et al., 2004).

1.3.4 Chloride channel toxins

So far only two chloride channel toxins have been identified: one is chlorotoxin, with a short peptide chain (36 residues) from the venom of scorpion *Leiurus quinquestriatus* (DeBin et al., 1993), and the other one is from the *Bm*K venom (to be addressed later). Chlorotoxin was considered to specifically target on chloride channels and was found to have specifically inhibitory effect on glioma (DeBin et al., 1993; Dalton et al., 2003). But it was found later that its inhibitory effect on glioma was by inhibiting matrix metalloproteinase 2, which was also up-regulated in glioma (Deshane et al., 2003). Because of the specific effect seen on glioma, chlorotoxin has been extensively studied for its potential therapeutic use (Lyons et al., 2002; Deshane et al., 2003).

1.3.5 Peptides not targeting on ion channels

The classes of scorpion toxins that do not target on ion channels include: (1) antimicrobial toxins; (2) bradykinin-potentiating peptides and (3) serine protease inhibitors. Antimicrobial toxins have no disulfide bridge. They are usually cationic or amphipathic small (2~5 kDa) peptides essentially having an alpha helical structure (Hwang and Vogel, 1998). A number of such peptides have been purified and characterized from scorpion venoms (Conde et al., 2000; Corzo et al., 2001; Moerman et al., 2002; Lee et al., 2004), and they can be provisionally classified into 6 subfamilies (Zeng et al., 2005). Probably due to their pore-forming ability, they have an immense potential in antimicrobial applications (Nomura et al., 2004; Nomura et al., 2005). Bradykinin-potentiating peptides also have no disulfide bridges. It was first

purified from the venom of *T. serrulatus* (Ferreira and Henrigques, 1992), and later identified in *Buthus occitanus* and *Buthus martentsi* scorpion venoms as well (Meki et al., 1995; Zeng et al., 2000). From the venom of scorpion *Tityus serrulatu,* a serine protease inhibitor, with the M.W. of 4,489 Da, was purified. It showed inhibitory activity against the rat plasma and urine kallikrein, and porcine pancreatic kallikrein (Ferreira et al., 1998).

1.4 Low M.W. toxins from the venom of *Bm*K scorpion

More than 70 peptides with various activities have already been discovered from the *Bm*K scorpion venom (Goudet at al., 2002). This venom contains all the ion channel toxins including sodium channel toxins, potassium channel toxins, chloride channel toxins and calcium channel toxin (Goudet et al., 2002; Cao et al., 2006). Additionally, it also has toxins without disulfide bond (Zeng et al., 2005).

*Bm*K toxins targeting sodium channels have been extensively studied. They are a group of toxins with relatively long peptide chains, consisting normally of 60~70 amino acids with 4 disulfide bridges. Thus far, more than 50 sodium channel peptides have been identified in *Bm*K venom (Goudet et al., 2002). Several sodium *Bm*K toxins were previously purified and characterized in our laboratory. Gong et al. (1997) isolated an α-neurotoxin named Makatoxin I from the venom of *Bm*K, and revealed that it had nitrergic activity, which would lead to a relaxant responses by the release of inhibitory neurotransmitter nitric oxide. Another toxin termed Chibutoxin, which was

subsequently isolated by our group (Gong et al., 1998), manifested adrenergic transmission inhibitory activity. Bukatoxin, an α-toxin purified from the venom of *Bm*K (Srinivasan et al., 2001) caused significant relaxant responses in the carbachol-precontracted rat anococcygeus muscle. A synthetic peptide (Buka11) representing the core potential activity domain of *Bm*K was examined, and found to be responsible for this relaxant activity.

The toxins targeting the potassium channels from *Bm*K venom have relatively shorter peptide chains consisting usually of 30~40 amino acids that are commonly stabilized by 3 disulfide bridges. Long chain potassium channel peptides containing more than 60 amino acids have also been found in the *Bm*K venom. A long chain potassium toxin BmP09 have been characterized (Yao et al., 2005) and two homologues have been cloned (Zhu et al., 1999). In total, more than 20 *Bm*K potassium toxins have been discovered so far (Goudet et al., 2002; Sheng et al., 2004; Xu et al., 2004a; Xu et al., 2004b; Yin et al., 2008).

Besides, chloride channel toxin or chlorotoxin homologues, calcium toxin, and toxins without disulfide bridge have also been found in the *Bm*K venom. Chlorotoxin homologues in *Bm*K venom have been reported years before but no function was described (Wu et al., 2000; Zeng et al., 2000; GenBank accession No.: AAK16444.1). Recently, a chlorotoxin-like neurotoxin has been successfully expressed and verified to have similar function as chlorotoxin (Fu et al., 2007). In 2006, the first calcium

toxin from *Bm*K venom, termed BmCa1, has been cloned and characterized. The precursor has 64 residues, and the mature peptide contains 37 amino acids (Cao et al., 2006). So far, four disulfide-bond-free toxins have been cloned from *Bm*K venom, with some tested to have antibiotic activities. (Zeng et al., 2004).

In sum, *Bm*K low M.W. toxins have been extensively studied resulting in a deeper understanding of structure-function relationship of ion channels, and a wider scope for the therapeutic potential. In fact, the toxins of *Bm*K venom are considered the most studied scorpion toxins in the world (Goudet et al., 2002). Their abundant source and marked significance attracted the researchers to focus on them and make an enormous contribution to related research fields. However, the emphasis on the small peptides probably attribute to the overlooking of large proteins from the same source. Prior to this work, no attempt has been made to address the issues of high M.W. proteins from the venom of *Bm*K.

1.5 High M.W. proteins from animal venoms

High M.W. proteins in the venom are mainly the water-soluble enzymes. The enzymes commonly found in venoms are hydrolases including proteinases, phosphodiesterases, exo-/endo-peptidases and phospholipases (Hider et al., 1991). Snake venoms are an important source for the venom enzymes and have been intensively studied. Enzymes have also been identified in other venomous sources like bees, scorpions, spiders and fishes. These enzymes include L-amino acid oxidase,

phospholipases A_2, serine protease, metalloproteinases, hyaluronidases, etc.. Other than these enzymes, some significant high M.W. non-enzymatic proteins also exist in animal venoms.

L-amino acid oxidase (LAAO):

LAAO is a flavoenzyme widely distributed in various organisms including animal venoms. In the presence of water and oxygen, it can catalyze stereospecific oxidative deamination of an L-amino acid substrate to ammonia, corresponding α-keto acid and hydrogen peroxide. LAAOs are the major components in many snake venoms and have been intensively studied. Most of the identified LAAOs have been enzymologically characterized (Du and Clemetson, 2002; Zhang and Wu, 2008), and some LAAOs, like the ones from the venoms of *Trimeresurus flavoviridis, Eristocophis macmahoni, Agkistrodon halys blomhoffii* and *Ophiophagus hannah* (Abe et al., 1998; Ali et al., 2000; Takatsuka, 2001; Jin et al., 2007) have been elucidated structurally. With the aid of X-ray crystallography, Pawelek et al. (2000) have revealed that LAAO from the venom of *C.rhodostoma* is functionally a dimer. Crystal structures of venom LAAOs from and *Agkistrodon halys pallas, Calloselasma rhodostoma* and *Vipera ammodytes ammodytes* (Zhang et al., 2004b; Moustafa et al., 2006; Georgieva et al., 2008) have also been established. LAAOs have various biological activities, including their marked inhibitory and activating effects on platelet function, induction of apoptosis, etc. (Du and Clemetson, 2002).

Phospholipase A_2(PLA_2)

PLA_2 is a common enzyme found in mammalian tissues as well as in animal venoms. Generally, PLA_2 specifically attacks sn-2 ester bond of phospholipids, and hydrolyzes it into arachidonic acid and lysophospholipid, which ultimately lead to many inflammatory processes. Hence, PLA_2 can be considered as inflammation upstream modulator (Dennis, 1994). However, it has rather broad biological activities; it can induce nerve growth, and has antibacterial activity, cytotoxicity and anticoagulation effect (Nevalainen et al., 2008; Makarova et al., 2007; Bonfim et al., 2006; Mounier et al., 2001). PLA_2 may fall into 3 subfamilies: secretory phospholipases A_2 ($sPLA_2$), cytosolic phospholipases A_2 ($cPLA_2$) and lipoprotein-associated PLA_2s (lp-PLA_2). All the venom PLA_2s are $sPLA_2$s, and their enzymological, pharmacological and even structural properties have been well characterized (Teixeira et al., 2003; Kini, 2003; Arni and Ward, 1996).

Serine protease

Serine proteases have a serine residue in the active sites of their molecules. Serine proteases have a variety of activities, and have been isolated and characterized from many animal venoms. Snake venom serine proteases have been found to possess both procoagulant and anticoagulant activities. Procoagulatant serine proteases, like the activators of factor V (Tokunaga et al., 1988), factor VII (Nakagaki et al., 1992), factor X (Tans and Rosing, 2001), prothrombin activators (Kini, 2005) and thrombin-like enzymes (Pirkle, 1998), as well as some anticoagulant proteases, like protein C

activators (Kisiel et al., 1987; Bakker et al., 1993) have been isolated from various snake venoms. Some of the snake venom fibrinolytic proteases (Zhang et al., 1995; Wisner et al., 2001) and kininogenase (kallikrein-like) proteins (Matsui et al., 1998) are also serine proteases. Besides their presence in snake venoms, serine proteases have also been found in the venoms of scorpions (Almeida et al., 2002) and spiders (Devaraja et al., 2008; Veiga et al., 2000), but the available data are rather limited as compared to the accumulated data available for snake venom proteases. Amidolytic activity test is a common assay to identify the serine protease from the venoms (Zhang et al., 1998a).

Metalloproteinases

Metalloproteinases, another member of the protease family, are commonly found in the spaces between cells, and hence are known as extracellular matrix metalloproteinase proteins (MMPs). As indicated by the name, a metal is needed for their activity. Most of them are zinc-dependent, and their activity can therefore be significantly inhibited by EDTA. Metalloproteinases are commonly found in animal venoms, and they exert various activities, amongst which the effect on the hemostatic system is the most significant. They fall into two classes: hemorrhagic and non-hemorrhagic. Both of them are fibrin(ogen)olytic enzymes, cleaving the Aα-chain in preference to the Bβ-chain (Markland, 1998). Snake venom metalloproteinases (SVMPs) also have the biological activities related to apoptosis (Wu et al., 2001; Masuda et al. 2001), proteolysis (Jeon and Kim, 1999), inflammation (Teixeira et al.,

2005) and inhibition of L-type Ca2+ channels (Zhang et al., 2009). All venom metalloproteinases that have been found so far are zinc-dependent enzymes with a Zn^{2+}-binding motif of HEXXHXXGXXH (Matsui et al., 2000). Many of them possess disintegrin domains as well (Ramos and Selistre-de-Araujo, 2006). Metalloproteinases have also been isolated and characterized in animal venoms other than those of snakes (e.g., in spider venom, Nagaraju et al., 2007), but the data are rather limited.

C-type lectin-like protein and Nerve growth factor

C-type lectin-like proteins are commonly found in snake venoms. They have the disulfide-bonding pattern similar to C-type lectins, but usually lack the calcium-dependent and sugar-binding properties of classical C-type lectins (Atoda et Morita, 1993). C-type lectin-like proteins mainly have the effect on platelet functions and hemostasis (Clemetson et al., 2005). It has also been isolated from the scorpion venom (Khoang et al., 2001).

Snake venom is one of the first sources available for isolation of NGFs, which are responsible for maintaining neurons and supporting their survival. Snake venom NGFs contain two identical or very similar subunits, and usually have the M.W. of more than 25,000 Da. As revealed by structural analysis, snake venom NGFs have amino acid sequences similar to those of the mammalian NGFs, (Kostiza and Meier, 1996). A recent study indicates that NGFs may facilitate the prey activity of venomous animals (Gennaro et al., 2007). NGFs are initially thought to be absent in the venoms of bees, scorpions, toads and spiders (Pearce, 1973) until Lipps (2000) has

successfully purified NGF from the honeybee venom. They are also known to be present in scorpion venoms (Lipps, 2000).

1.6 Hyaluronidase and its substrate hyaluronan (formerly hyaluronic acid)

Hyaluronidases are a group of neutral- and acid-active enzymes found throughout the animal kingdom in organisms as diverse as microbes, bees, wasps, hornet, spiders, scorpions, fish, snakes, lizards, and mammals (Kemparaju and Girish, 2006; Stern and Jedrzejas, 2006). They can hydrolyze the substrate hyaluronan, which is found predominantly in connective tissues, skin, cartilage, and in synovial fluid of mammals (Delpech, 1997). Hyaluronidases could be divided into three groups according to their action mechanisms: 1) endo-β-N-acetyl-hexosaminidases group 1, EC 3.2.1.35 (mainly found in vertebrates); 2) endo-β-N-acetyl-hexosaminidases group 2 EC 4.2.99.1 (mainly found in bacteria); and 3) endo-β-glucuronidases EC 3.2.1.36 (hyaluronidases from leech and crustaceans). The first two classes are well studied, but the third class remains little known. In human proteomics, there are 5 hyaluronidases expressed: Hyaluronidase-1 to -4 and PH-20. Except for PH-20, which mainly associates with the testes, the first 4 homologues distribute widely in the body. The other important source of hyaluronidases is animal venoms. Hyaluronidases act as a "spreading factor" in animal venoms to facilitate the penetration of other toxins during the envenomation (Duran-Reynals, 1933).

In bacteria, all the bacterial hyaluronidases are lyases. Beacteria employ their

hyaluronidases for two purposes: 1) to overcome the defense system of the host by degrading hyaluronan, one of the major components of the tissue extracellular matrix (ECM); 2) to make use of disaccharide, the degradation product that can provide the bacteria with the carbon and energy (Stern and Jedrzejas, 2005).

As the substrate of hyaluronidases, hyaluronan is a linear polysaccharide linked by as many as 25,000 repeating units of disaccharides (D-glucuronic acid and D-N-acetylglucosamine) (Fig. 1.4). It is the primary composition of ECM, and its degradation and synthesis are in dynamic equilibrium at any moment. Degradation of hyaluronan by hyaluronidase is accomplished by cleavage at either β1,3 glycosidic bond or β1,4 glycosidic bond, but the cleavages at β 1,4 glycosidic bond are much more common (Stern and Jedrzejas, 2006). Different hyaluronidases may produce the digestion product in different sizes. The resulting fragments interact with different receptors of the cell membrane and induce correspondingly downstream physiological/pathological events, such as anti-angiogenesis, cell invasion/motility and wound repair (Tempel et al., 2000; Sugahara et al., 2006; Dechert et al., 2006).

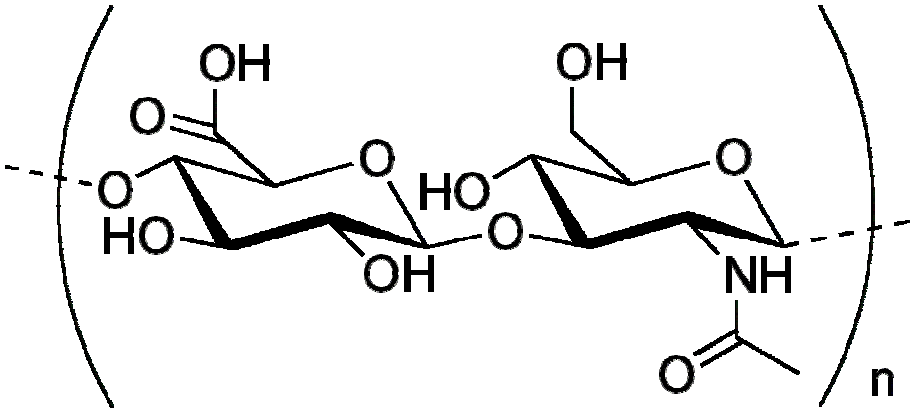


Fig. 1.4. Chemical structure of hyaluronan. Hyaluronan polymer is composed of the repeating units of glucuronic acid and *N*-acetylglucosamine. n: 20~25,000. Freely licensed image from Wikipedia.

1.7 Venom hyaluronidases

So far, a number of hyaluronidases have been isolated from the venoms of bees, snakes, wasps, hornets, spiders and fish (Gmachl and Kreil, 1993; Xu et al., 1982; Kudo and Tu, 2001; Girish et al., 2004; King et al., 1996; Lu et al., 1995; Wright et al., 1973; Poh et al., 1992). Hyaluronidase activity has been identified in the brown recluse spider venom by Wright et al. (1973), and a hyaluronidase was purified and partially characterized from the venom of the snake *Agkistrodon acutus* by Xu et al. (1982), which was a glycoprotein with a M.W. of 33 kDa. Investigation of the M.W.s of a number of venom hyaluronidases using SDS-PAGE demonstrated that the venom samples from different animals - *Dolichovespula maculata* (whitefaced hornet), *Yespula germanica* (a yellow jacket), *Pogonomyrmex rugosus* (harvester ant),

Heloderma horridum horridum (Mexican beaded lizard), *Heloderma suspectum suspectum* (Gila monster), *Lachesis muta* (bushmaster snake), *Crotalus basiliscus* (Mexican west-coast rattlesnake), *Bothrops riper* (Central American pit viper), *Micsurus nigrocinctus* (a Central American coral snake) and *Centruroides limpidus limpidus* (Mexican west-coast scorpion) - contain the hyaluronidases. The hyaluronidases from all invertebrates are found to have smaller M.W.s (< 50 kDa) than those of vertebrates, which usually had M.W.s of over 60 kDa. The first marine source of hyaluronidase was reported by Poh et al. (1992), who purified a hyaluronidase with a *p*I of 9.2 and a M.W. of 62 kDa from the venom of stonefish (*Synanceja horrida*). A hyaluronidase from the white face hornet (*Dolichovespula maculata*) venom was identified by cloning and expression in bacteria (Lu et al., 1995). As manifested by sequence comparisons, it was found to have 56% sequence identity with the honeybee venom hyaluronidase, and 27% identity with human sperm PH-20. A hyaluronidase from the venom of yellow jacket wasp (*Vespula vulgaris*) was also cloned and expressed, using the RACE PCR technique (King et al., 1996). Although a number of snake venom hyaluronidases were reported previously (Kudo and Tu, 2001; Kemparaju and Girish, 2006), no structural information had been given, except for a short 10-amino-acid N-terminal sequence provided by Girish et al. (2004), until Harrison et al. (2007) cloned the first snake venom hyaluronidase gene. The most studied hyaluronidase should be the one from the venom of honeybee (*Apis mellifera*), which was purified and partially sequenced by Kemeny et al. (1984). Based on this result, Gmachl and Kreil (1993) cloned its cDNA and found the

deduced protein to be homologous to the guinea pig sperm PH-20. The mature bee venom hyaluronidase has 349 amino acids containing three potential *N*-glycosylation sites. Following expression in *E.coli,* the recombinant protein was found to have retained the activity. The crystal structure of bee venom hyaluronidase, the only experimental structure available for hyaluronidase, was elucidated by Marković-Housley et al. (2000).

Besides the bee venom hyaluronidases, a few hyaluronidases have also been identified in scorpion venoms. The isolation of this enzyme has been described previously in the venom of the scorpion *Heterometrus fulvipes* by Ramanaiah et al. (1990). Other scorpion hyaluronidases have been isolated and characterized from the venoms of *Tityus serrulatus* (Pessini et al., 2001) and *Palamneus gravimanus* (Morey, 2006). Most recently, the first N-terminal sequence of the scorpion hyaluronidase from the *Tityus stigmurus* venom has been reported by Batista et al. (2007) through a proteomic investigation of the venom from scorpion. However, this 'hyaluronidase' enzyme has not been characterized and the sequence was found later to be dubious (to be addressed in "Discussion"). Before the present work, no full sequence has been revealed for the scorpion venom hyaluronidases, and their structures are totally unknown.

A summary on venom hyaluronidases is listed in Table 1.1.

1.8 Structures of hyaluronidases

A number of hyaluronidase protein sequences have been established. Of human origin, there are five hyaluronidase proteins: Hyaluronidase-1~-4 and PH-20, all of which have a high degree of homology. Sequence identities among the human hyaluronidases are from 33.1%, which is noted between the human Hyaluronidase-3 and -4, to 41.2%, between the human Hyaluronidase-4 and human PH-20 (Jedrzejas and Stern, 2005). The structure of the hyaluronidase enzymes are tightly related to their function. Detailed studies have been conducted for the bee venom hyaluronidase (BVHYA) and bovine PH-20 (BPH-20). Both BVHYA and BPH-20 are globular proteins containing a large cleft which allows the binding of the substrate for catalysis. A classical distorted $(\beta/\alpha)_7$ triose phosphate isomerase (TIM) barrel fold, typically presenting in all the glycoside hydrolases of carbohydrate active enzyme family 56, is manifested in the structures of BVHYA and BPH-20. The clefts in these two hyaluronidases can accommodate the entire hexasaccharide fragment of hyaluronan. Negatively charged hyaluronan chains, which also have hydrophobic sugar ring surfaces, can readily bind to the cleft whose surface is lined with positive charged/hydrophobic amino acid residues for subsequent catalysis process (Fig.1.5.). H-donor catalytic Glu residue, which is encompassed by a number of positioning residues, is strictly conserved in all the known hyaluronidases. Glu^{113} in BVHYA and Glu^{149} in BPH-20, including the positioning residues are highly conserved, and any structural changes may lead to functional alterations. For example, removal of the Cys^{264} residue, one of the positioning residues of human hyaluronidase-4, may cause

alterations in the specificity of this enzyme (Table 1.2).

Table 1.1. Biochemical and biophysical properties of animal venom hyaluronidases (modified and updated from Kemparaju and Girish, 2006, to be continued on next page)

Source	M.W. (kDa)	*p*I	Optimum pH	Optimum Temp. (^{o}C)	Kinetic para. K_m (μg/mL); V_{max} (μg/min)	Final digested hyaluronan fragment	Structural info.	Ref.
A. acutus (snake)	33	10.3	3.5~5.0	37	6.2; N.D.	N.D.	N.D.	Xu et al.,1982
A.contortrix contortrix (snake)	59.3	9	6	37	N.D.	Tetrasaccharide	N.D.	Kudo and Tu, 2001
N.naja isoform 1	70.4	9.2	5	37	N.D.	Tetrasaccharide	10 a.a. of N-terminal	Girish et al., 2004
isofrom 2 (snake)	52	9.7	5	37	N.D.	N.D.	N.D.	Girish and Kemparaju, 2005
H.fulvipes (scorpion)	82	N.D.	4	N.D.	N.D.	N.D.	N.D.	Ramanaiah et al.,1990
T.serrulatus(scorpion)	51	N.D.	6	40	69.7; 3.3	N.D.	N.D.	Pessini et al., 2001
A.mellifera (bee)	41	9	6	37	N.D.	Tetrasaccharide	Full primary and 3-D	Kemeny et al., 1984; Gmachl, and Kreil; 1993; Marković-Housley et al.,2000
S.horrida (fish)	62	9.2	6	37	709;13.1	N.D.	Full primary	Poh et al., 1992; Ng et al., 2005
H.horridum horridum (lizard)	63	5	5	Sensitive	N.D.	N.D.	N.D.	Tu and Hendon, 1983
L.obliqua isoform1	49	N.D.	6~7	37	N.D.	N.D.	N.D.	da C B Gouveia et al., 2005
isoform2 (caterpillar)	53	N.D.	6~7	37	N.D.	N.D.	N.D.	da C B Gouveia et al., 2005
L.reclusa isoform1	33	N.D.	5~6.6	37	N.D.	N.D.	N.D.	Wright et al., 1973
isoform2 (spider)	63	N.D.	5~6.6	37	N.D.	N.D.	N.D.	Wright et al., 1973

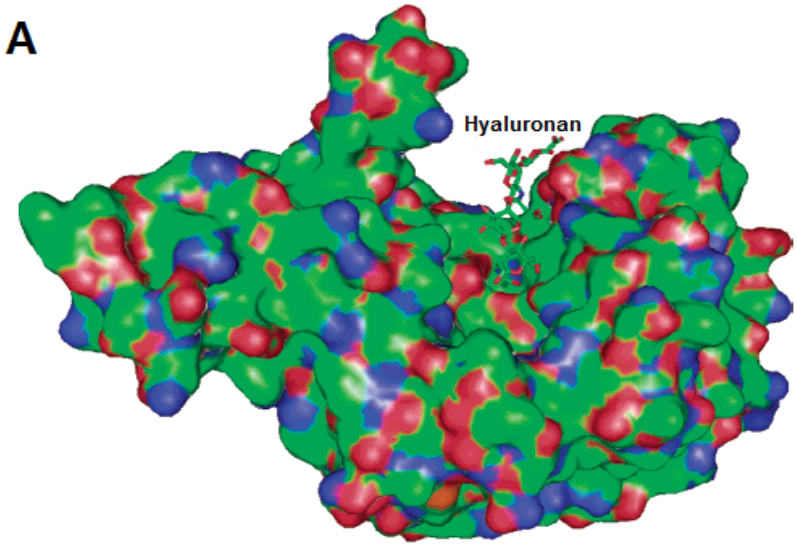
Table 1.1.(continued) Biochemical and biophysical properties of animal venom hyaluronidases (modified and updated from Kemparaju and Girish, 2006)

Source	M.W. (kDa)	*p*I	Optimum pH	Optimum Temp. (^{o}C)	Kinetic para. K_m (μg/mL); V_{max} (μg/min)	Final digested hyaluronan fragment	Structural info.	Ref.
Palamneus gravimanus(scorpion)	52	N.D.	4.5	37	47.61;1.49	N.D.	N.D.	Morey et al., 2006
Tityus stigmurus(scorpion)	44.8	N.D.	N.D.	N.D.	N.D.	N.D.	24 a.a. of N-terminal	Batista et al., 2007
Echis ocellatus(snake)	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	Full primary	Harrison et al., 2007
Potamotrygon motoro (fish)	79	N.D.	4.2	40	4.91; 2.02*	N.D.	N.D.	Magalhães et al., 2008
Vitalius dubius (spider)	45	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	Rocha-e-Silva et al., 2009
Loxosceles intermedia isoform 1	41	N.D.	6~8	N.D.	N.D.	N.D.	N.D.	da Silveira et al., 2007
isoform 2 (spider)	43	N.D.	6~8	N.D.	N.D.	N.D.	N.D.	da Silveira et al., 2007
Hippasa partita (spider)	42.26	N.D.	5.8	37	N.D.	N.D.	N.D.	Nagaraju et al.,2007
BmHYA1	48.7	8.65 **	4.5	50	95.3; 3.9	Tetrasaccharide	Full primary	Present work

*: unit of U/min; **: calculated; N.D.: not determined; a.a.: amino acid residues.

Table 1.2. Numbering scheme for conserved residues of the hyaluronidases involved in the catalytic process, and in essential positioning of the substrate's carbonyl of the acetamido group for catalysis (Jedrzejas and Stern, 2005). The Cys^{263} residue of human hyaluronidase-4 interrupts the conserved scheme, which may reflect this human hyaluronidase-4's specificity for chondroitin and its chondroitinase activity (Modified from Stern and Jedrzejas, 2006). HHYA-1~4: human hyaluronidase-1~4.

Hyaluronidases	BVHYA	BPH-20	HHYA-1	HHYA-2	HHYA-3	HHYA-4
Catalytic residue	Glu113	149	131	135	129	147
Positioning residues	Asp111	147	129	133	127	145
	Tyr184	220	202	206	202	218
	Tyr227	265	247	253	246	Cys^{263}
	Trp301	341	321	327	319	339



(A) The X-ray structure of native BVHYA showing binding of the tetra-saccharide hyaluronan to the surface of the enzyme within the cleft. The protein surface and the substrate hyaluronan are color-coded by atomic element. The color denotes: green-C, purple-N, red-O and yellow-S.

(B) The distorted $(\beta/\alpha)_7$ TIM barrel fold and the cleft are shown. The molecules (both enzyme and substrate) are color-coded by the secondary structure elements (red-helices, yellow-β-sheets, green-other elements). The hyaluronan bound to the enzyme is located in the cleft (depicted in ball-and-stick fashion and colored in magenta).

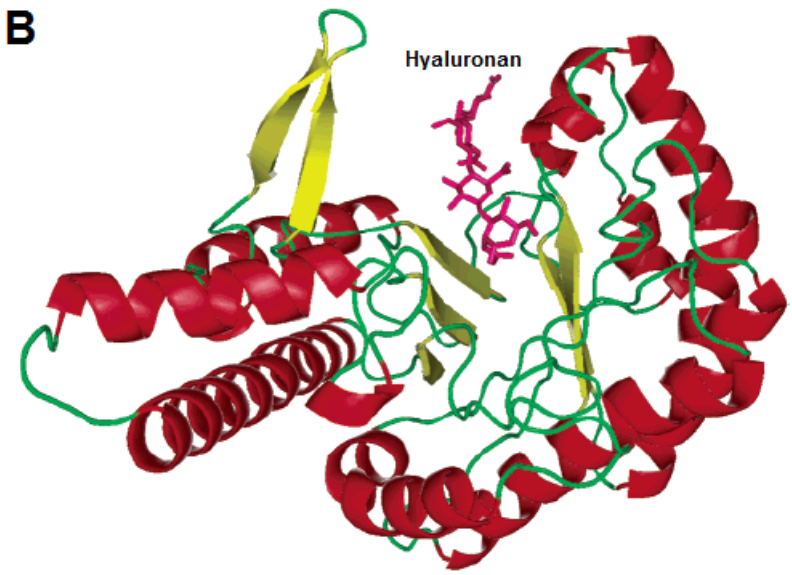


Fig.1.5. Tertiary structure of BVHYA with a hyaluronan tetrasaccharide (From Stern and Jedrzejas, 2005). Citation with permission by American Chemical Society (License number: 2304270071837)

1.9 The biological and medical applications of hyaluronidases

With a view to use venoms as therapeutic potentials for medical applications, previous studies have focused more on the toxic components of the venom rather than on the non-toxic components like hyaluronidases (Kreil, 1995). As mentioned earlier (section 1.6), hyaluronidases, as well as the degraded hyaluronan fragments, have been shown to have interesting biological effects not only on anti-angiogenesis, cell invasion/motility and wound repair (Tempel et al.,2000; Sugahara et al., 2006; Dechert et al., 2006), but they are also known to play an important role in cancer chemotherapy of solid tumors. Application of hyaluronidase in chemotherapy may enhance the immunological control of the cancers by degradation of hyaluronan, especially for those cancers which are coated with abundant CD44-bound hyaluronan (Spruβ et al., 1995).

The significance and applications of hyaluronidase in cancer therapy has been long recognized by the biomedical reserachers and clinicians alike. In a number of pilot clinical studies, it was previously reported that the use of hyaluronidases as an adjunct to the conventional chemotherapy significantly improved the prognosis of most patients. In some cases, the supplemental treatment with the enzyme even induced significant responses in previously chemoresistant patients. (Beckenlehner et al., 1992; Smith et al., 1997; Klocker et al., 1998). It was assumed that the degradation of hyaluronate-enriched ECM by the hyaluronidases might have facilitated the drug to readily exert its action on the target. Beckenlehner et al., (1992) studied hyaluronidase effect on the activity of adriamycin in breast cancer models both *in vitro* and *in vivo*. The activity of adriamycin was found to be significantly improved both *in vitro* and *in vivo.* It was speculated that

hyaluronidase facilitates the diffusion of adriamycin by making a hydrated space in the cells. Tumor growth was then indirectly inhibited by enhanced accessibility of the chemotherapeutic. It was also found by Shuster et al. (2002) that the exogenously added hyaluronidase repressed tumor growth via hydrolyzing the hyaluronan halo surrounding the tumor cells, and it was speculated that CD44 may be involved in this process. CD44 is the cell-adhesion molecule widely distributed throughout the cell membrane that is involved in a number of biological events such as cell adhesion, cancer cell invasion and migration (Naor et al., 1997). CD44-hyaluronan interaction is important to cancer growth and progression (Platt and Szoka, 2008). The interfering of CD44-hyaluronan interaction by hyaluronidase, via disruption of hyaluronan matrix, has been found to effectively decrease the tumor growth (Shuster et al., 2002). Sy et al. (1992) reported that the soluble CD44-immunoglobulin fusion protein could suppress the tumor growth formed by human Burkitt lymphoma cells transfected with CD44. Zeng et al., (1998), on the other hand, demonstrated that the formation of tumor by B16F10 melanoma cells could be inhibited by hyaluronan oligomers. It is also likely that some tumors themselves may express hyaluronidase for their invasion (Udabage et al., 2005; Lokeshwar et al., 2005). It seems hyaluronidase can be both the promoter and suppressor for tumors. The role hyaluronidases play in cancer biology is still in question (Lokeshwar and Selzer, 2008).

Hyaluronidases may also show indirect effects by the degraded hyaluronan fragments, which are in fact polysaccharides in different sizes. The high M.W. hyaluronan fragments (>400 kDa) play a role in anti-angiogenic, anti-inflammatory and immunosuppressive, while the medium fragments (~20 kDa containing ~50 saccharide

units) can give rise to the synthesis of inflammatory cytokines. The low M.W. fragments (6~20 kDa), on the other hand, may stimulate the dendritic cells and antigen presenting cells in the immune system and may thus have an effect on angiogenic, inflammatory and immuno-stimulatory processes (Stern, 2005). Relatively smaller hyaluronan degradation fragments containing 3-10 disaccharides are also detected among the degradation products of hyaluronidases. This group of hyaluronan fragments inhibits anchorage-independent growth of tumor cells. For instance, tetrasaccharides, the main products of human HYA-1, have anti-apoptotic activities, suppressing cell death in cultured cells undergoing hyperthermia or serum starvation (Stern, 2005). The high M.W. fragments are often present in normal biological process, while the low M.W. fragments show up frequently in cancers (Kumar et al., 1989; Lokeshwar et al., 1997). The fragments of hyaluronan play a role via interaction with the cell membrane receptors like CD44 and RHAMM. For example, proteolytic cleavage of CD44 by the low M.W. hyaluronan fragments can enhance the motility of tumor cells (Stern et al., 2006).

1.10 Aims of the present study

The purpose of the present study is to investigate the high M.W. constituents of the venom from *Bm*K, particularly the hyaluronidase enzyme, by purification, characterization, molecular cloning and expression, and to study its potential for medical applications.

The primary objectives of this study are:

1) To screen for the biological activities of *Bm*K crude venom. The candidate activities include LAAO, serine protease, fibrinolytic, hemolytic, hyaluronidase and antibacterial.

2) To purify and characterize the hyaluronidase from the venom of *Bm*K.

3) To describe and compare the enzyme characteristics. (i.e., optimal temperature, optimal pH, thermal stability, etc.), and to determine/analyze its N-terminal amino acid sequence, M.W. and final digestion product.

4) To analyze the divergence and evolution of BmHYA1 through cloning and molecular characterization of its cDNA and comparison of BmHYA1 with other known hyaluronidase protein sequences, since it is supposed to be the first full protein sequence from scorpion species.

5) To establish an *E.coli* expression system for production of the recombinant *Bm*K hyaluronidase protein, in order to produce enough protein for future studies, e.g., functional and structural studies.

6) To study the modulatory effects of *Bm*K hyaluornidase on CD44 modulation in MDA-MB-231 breast cancer cell line, exploring its potential therapeutic applications.

CHAPTER II

MATERIALS AND METHODS

2.1 The venom

The lyophilized *Bm*K crude venom (BCV) was bought from a supplier in Zhejiang Province, P.R. China. The animal was stimulated by an electric instrument and collected manually in a clean and chilled beaker. The wet venom was frozen and lyophilized after it was secreted. One gram of dried scorpion venom was obtained from about 3,000 scorpions. The lyophilized BCV was light yellow colored crystals. The crystals were weighed and dissolved in 0.05 M ammonium bicarbonate (pH 8.9), and then centrifuged at 50,000 $\times g$ for 20 minutes to give a 100 mg/mL of BCV solution. The precipitated palette was removed and the suspended jelly-like materials were sieved by 0.2 μm filter. The transparent yellow solution, with the final protein concentration of 0.34 mg/mL, as determined by Bio-Rad protein assay kit (Hercules, CA, USA), was stored at 4 ^{o}C until use.

2.2 Gel filtration of BCV and M.W. distribution of fractions

Gel filtration chromatography was used for the preliminary separation of BCV, as it separates the samples according to their M.W., which is apparent and in consistent with the previous basis for classifying the protein components in venoms, i.e., the M.W. - the high M.W. proteins and low M.W. peptides. The prepared BCV solution was loaded onto the Superdex G75 gel filtration column (Hi-load 16/60, Pharmacia Biotech, Uppsala, Sweden), and the venom fractions eluted with 50 mM ammonium bicarbonate, pH 8.9, at a flow rate of 1 mL/min. The absorbance was monitored at 280, 254, and 215 nm simultaneously. The protein concentration of

eluted fractions was determined by Bio-Rad protein assay kit (Hercules, CA, USA). The M.W. distribution was investigated by SDS-PAGE for high M.W. components, and mass spectrometry for low M.W. components. Subsequently, BCV and its respective fractions were tested for a variety of activities as follows.

2.3 Screening of the biological activities of BCV and its gel filtration fractions

LAAO activity:

The assay was conducted in a 96-well microplate in triplicate. To initiate the reaction, 10 μL of crude venom solution and 90 μL of substrate solution were added into each well. The standard reaction mixture contained 5 mM of L-Leucine (substrate), 2 mM μ-phenylenediamine (OPD), 0.81 U/mL horseradish peroxidase (HRP) and either the test venom solution or the snake *Naja Kaouthia* crude venom solution (positive control) as LAAO source, in a total volume of 100 μL/well of 50 mM Tris–HCl buffer (pH 8.0). For negative control, 50 mM ammonium bicarbonate, pH 8.9 was added into the well in place of the sample or standard LAAO. After incubation at 37 ^{o}C for 60 minutes, the reaction was terminated by adding 50 μL of 2 M H_2SO_4. The absorbance of the reaction mixture was measured at 450 nm (Kishimoto and Takahashi, 2001).

Fibrinogenolytic activity:

Fibrinogenolytic activity was measured as follows: samples (from 10 peak

fractions) and human fibrinogen (5 mg/mL) dissolved in 50 mM Tris–HCl buffer, pH 7.4, containing 0.1 M NaCl and 10 mM $CaCl_2$, were incubated at 37 ^oC for 16 hours. After the incubation, 20 μL of the reaction mixture was transferred into a small test tube, to which 20 μL of the solution containing 1% sodium dodecyl sulfate (SDS) and 2% β-mercaptoethanol was added. The resultant solutions were loaded onto 15% SDS-PAGE gel. The degradation of the fibrinogen chains indicated the existence of the activity (Gao et al. 1998).

Hemolytic activity

Hemolytic activity was assayed on citrated rat blood and washed erythrocytes (Gao et al, 1999). One hundred and ninety ul of erythrocyte suspension (washed for three times with 20 mM Tris–HCl, pH 7.4, containing 1 mM $CaCl_2$ and 0.9% NaCl and resuspended for 10 times with the same buffer) were incubated with the samples (10 μL) at 37 ^oC for 30 minutes. The degree of hemolysis was determined as the percentage of hemoglobin released (measured at 540 nm) by the toxin compared with a 100% hemolysis produced by the addition of 1% Triton X-100. For the whole blood, no washing was performed, and the blood (collected in 3.8% sodium citrate, 1:9, v/v) was directly added with the samples and incubated at 37 ^oC for 30 minutes. Positive control (containing 1% Triton X-100) was the same as that used for the erythrocyte test. Ammonium bicarbonate (50 mM, pH 8.9) buffer alone without the samples was used as a negative control.

Antibacterial activity:

Antibacterial activity was assayed by the method of agar-well diffusion assay (Mathabe et al., 2006) with modification, by observing the growth inhibition of *Escherichia coli* DH5α. The agar plates were prepared by pouring approximately 15 mL of molten media (1% Tryptone, 0.5% yeast extract, 1% sodium chloride and 1.5% agar, all from Sigma, St. Louis, MO, USA) into sterile petriplates (diameter=90 mm, Sterilin, Middlesex, UK), which were allowed to solidify for 20 minutes. The inoculum suspension of *E. coli* (100 μl in frozen glycerol stock, for preparation see section 2.6.4.3) was swabbed uniformly on the agar plates, and the inoculum was allowed to dry for 5 minutes. Since it is only a preliminary screening test, instead of making a well in the agar, the samples (i.e., BCV and its fractions) at various quantities (10 μg~ 1000 μg), were put directly on the bacterial colonies in pre-determined regions on the agar when the inoculum was dried. The plate was then put into 37 ^{o}C incubator overnight. If the bacterial growth was inhibited, no bacteria would grow on the agar where the sample was applied. The antibacterial activity was shown as a round inhibition zone appearing on the agar.

Amidolytic activity:

Amidolytic activity assay was followed by the method of Zhang et al.(1998b). Chromogenic substrate S-2238 (d-Phe-(pipecolyl)-Arg-*p*-nitroaniline hydrochloride) was used for the test. Assays were performed in 50 mM Tris–HCl, pH 7.8, 0.01% Tween-80 in a total volume of 500 μL. The reaction was launched by the addition of

the sample and the formation of *p*-nitroanilide (*p*NA) was monitored continuously at 405 nm. The amount of substrate hydrolyzed was calculated from the absorbance at 405 nm by using a molar extinction coefficient of 10,000 M^{-1} cm^{-1} for free *p*NA.

PLA_2 activity:

The PLA_2 activity was assayed using Secretory Phospholipase A_2 Assay Kit (Cayman Chemical, Ann Arbor, MI, USA). $sPLA_2$ activity was tested in alternate fractions collected from tubes 41 to 79 (mL). Ten microliters of elution fractions were added to the wells together with 10 μL of DTNB and 5 μL of assay buffer (both provided by the kit). The reaction was initiated by adding 200 μL of substrate. ΔA_{405}/min was determined and $sPLA_2$ activity ($\mu mol*min^{-1}*mL^{-1}$) was calculated using the formula indicated in the manual.

Hyaluronidase:

Hyaluronidase enzyme activity was determined turbidometrically by the method of Poh et al. (1992). Hyaluronic acid (0.5 mg/mL in 0.05 M acetate buffer, pH 4.5, containing 0.15 M NaCl) was incubated with the test solution for 15 minutes at 37 ^oC (final volume 0.5 mL) and the reaction was stopped by the addition of 1 mL of 2.5% (w/v) hexadecyltrimethylammonium bromide in 2% (w/v) NaOH. The absorbance of each reaction mixture was read at 400 nm. Turbidity reducing activity was expressed as the percentage of the remaining hyaluronan. The absorbance was taken as 100% in which no enzyme was added. Turbidity reducing units are expressed as the amount of enzyme needed to hydrolyze 50% of 50 μg hyaluronic acid. One unit was defined as

the amount of enzyme that would cause the same turbidity reduction as 1.0 unit of international standard preparation. The blank consisted of one volume of acetate buffer (0.05 M acetate buffer, pH 4.5, containing 0.15 M NaCl) and two volumes of stop solution.

2.4 The purification of BmHYA1

2.4.1 Gel filtration

The crude venom was initially subjected to gel filtration chromatography as described in section 2.2.

2.4.2 Anion exchange chromatography

The pooled fractions collected in the previous step were applied on UNOQ1 anion exchange column (7×35 mm, Bio-Rad, Hercules, CA, USA) pre-equilibrated with 50 mM NH_4HCO_3 buffer, pH 8.9. The samples were eluted with a 0 ~ 40% 1 M NaCl gradient, at a flow rate of 1.5 mL/min. The purification process was monitored at the absorbance of 215 nm. The eluted fractions with hyaluronidase activity were pooled and lyophilized for further purification.

2.4.3 Cation exchange chromatography

The lyophilized pooled fraction was then loaded onto the UNOS1 cation exchange column (7×35 mm, Bio-Rad, Hercules, CA, USA). The column was equilibrated with 50 mM sodium acetate, pH 5.0, and the fractions eluted by applying

an increasing gradient of 0 to 100% NaCl, at a flow rate of 1.5 mL/min. The purification process was monitored at the absorbance of 215 nm.

2.4.4 RP-HPLC

For final purification of the target enzyme, the RP-HPLC C_8 column, pre-equilibrated with 0.1% TFA, was used at a flow rate of 0.5 mL/min. The purification process was monitored at the absorbance of 215 nm. The increasing gradient of acetonitrile was applied to elute the target protein, which displayed a single peak upon elution, and later verified to have the hyaluronidase activity in the enzyme assay.

2.5 Characterization of BmHYA1

2.5.1 SDS-PAGE

Vertical SDS-PAGE gel electrophoresis is a widely-used technique for analyzing protein molecules. Liquid acrylamide molecules polymerized and form a net-like gel when solidified. Under an electric field, protein which carries negative-charged SDS molecules, will migrate towards the positive electrode. The smaller of the protein molecule, the faster it can move. In this experiment, SDS-PAGE mini gel was used for confirmation of the homogeneity of the enzyme and estimation of its M.W.. The sample obtained from the final chromatographic (RP-HPLC) step was subjected to 12% SDS-PAGE gel with Tris-Glycine buffer at pH 8.8 as a running buffer. The gel was then developed in a suitable volume of GelCode Blue Stain Reagent (Thermo Scientific, Waltham, MA, USA) for 1 hour and washed with MilliQ water.

2.5.2 Mass spectrometry

MALDI-TOF MS is a common and sensitive way to determine the precise M.W. of proteins and peptides. The measurement was carried out at the Protein and Proteomics centre, National University of Singapore, by MALDI-TOF (ABI 4700 linear mode) mass spectrometry.

2.5.3 N-terminal sequencing

The technique for the determination of protein N-terminal amino acid sequence, as developed by Edman (Edman, 1950), is involved in a phenylisothiocyanate (cyclic) degradation procedure. Amino acid residues are cleaved once a time from the N-terminal of the protein and identified as the phenylthiohydantion derivatives. ABI Procise 494 Protein Sequencer at the Protein and Proteomics Centre of Department of Biological Science, National University of Singapore, was used to conduct Edman degradation to determine the N-terminal sequence of the purified enzyme.

2.5.4 Optimal pH and temperature

Suitable amounts of the enzyme were used for examining the relative activity under various conditions. The maximum activity was set as 100% and other degrees of activity were expressed as different percentages compared with the maximum one. Different buffer systems - citrate buffer for pH 3 and below, acetate buffers for pH 4-6, phosphate buffer for pH 7 and Tris-HCl buffers for pH 8 and above - were used to

study the optimal pH. In another series of experiments, various temperature settings – 4, 24 (room temperature), 37, 45, 50, 55, 60, 70 and 80 ^{o}C – were used to study the temperature effect. The amount of each of the three enzymes (BmHYA1, bovine testicular hyaluronidase and *Daboia russelii* snake venom hyaluronidase) was adjusted to degrade approximately 50% of the hyaluronan (25 μg) in 15 minutes at 37 ^{o}C. The reaction buffer was 50 mM sodium acetate pH 4.5.

2.5.5 Thermostability

The stability of the enzyme with respect to temperature was measured by the method of Zaneveld et al.(1973). The hyaluronidase was pre-incubated at different temperatures for 10 minutes in a test tube, and then immediately cooled in tap water before incubating the tube (containing the whole reaction system, see section 2.3: Hyaluronidase) in a 37 ^{o}C water bath for 15 minutes.

2.5.6 K_m and V_{max} determination

K_m and V_{max} are two terms involved in enzyme kinetics. K_m denotes 1) the affinity of substrate to the enzyme and 2) the rate at which the complex converts to the product after enzyme-substrate binding. V_{max} refers to the maximum initial velocity of the enzyme catalyzed reaction under a given condition, which occurs when the enzyme is saturated by the substrate (i.e., the enzymatic sites are all occupied by the substrate). Both of the two constants cannot be measured directly. The Michaelis-Menten equation can be used to calculate the two parameters. The determination of

K_m and V_{max} was by the method of Poh et al. (1992) with modification. The amount of hyaluronidase was adjusted to hydrolyze 50% of 50 μg hyaluronan in 15 minutes under 37 ^{o}C and at pH 4.5. Under the same condition, the increasing amounts of the hyaluronan (10~35 μg) were added into the reaction system for incubation. The velocity of the hydrolysis was expressed by the hyaluronan deposition at microgram level.

2.5.7 Deglycosylation of BmHYA1

Deglycosylation test was performed using Enzymatic Protein Deglycosylation Kit (Sigma, St. Louis, MO, USA), in order to study the glycosylation type of BmHYA1. Thirty micrograms of BmHYA1 was dissolved in 30 μL of deionized water in an Eppendorf tube. Ten microliters of 5X Reaction Buffer and 2.5 μL of Denaturation Solution were added, following which the solution was gently mixed and heated at 100 °C for 5 minutes. After the reaction system was cooled to room temperature, TRITON X-100 solution (2.5 μL) was added, followed by addition of 1 μL each of the PNGase F, O-Glycosidase, and α-2(3,6,8,9) Neuraminidase. The reaction solution was then incubated for 24 hours at 37 °C. The result was analyzed by 15% SDS-PAGE. The deglycosylated protein had relatively low M.W. and would shift to a lower position. According to the product manual, PNGase F cleaves most asparagine-linked complex, hybrid, or high mannose oligosaccharides; α-2(3,6,8,9) Neuraminutesidase cleaves all non-reducing terminutesal branched and unbranched

sialic acids; and O-Glycosidase cleaves serine or threonine-linked unsubstituted Gal-β(1-3)-GalNAc-α−.

2.5.8 Effect of inhibitors on hyaluronidase activity

The hyaluronidase concentration was adjusted to the extent that the turbidity was reduced to 10% of the original. The hyaluronidase potential inhibitors, i.e., Mg^{2+}, Ca^{2+}, Cu^{2+}, Fe^{3+}, reduced glutathione, L-cysteine, DTT, EDTA and heparin, were assayed for their inhibitory activity. They were pre-incubated with the enzyme for 15 minutes at 4 ^{o}C. Then the hyaluronan was added to initiate the reaction at 37 ^{o}C for 15 minutes. Control experiments were carried out with no inhibitors added or the inhibitors added only immediately before the measurement to counteract the color caused by the inhibitors themselves.

2.5.9 Thin-layer chromatography (TLC) for determination of the final digestion product

TLC is a kind of chromatography to separate the mixed samples in a thin layer of adsorbent material, which was the silica gel in this experiment. The resultant digestion samples contained high concentration salt and therefore could not be applied directly to MS to test their M.W.. A method of (Kudo and Tu, 2001) with modification was used for analyzing the final digestion product of hyaluronan by BmHYA1 and bovine testicular hyaluronidase. The digestion product of hyaluronan by bovine testicular hyaluronidase, which is known to produce tetrasaccharides (Kudo and Tu, 2001), was

used as a marker for molecular size comparison. Two hundred micrograms of substrate and 700 units of enzymes were mixed in 50 mM sodium acetate buffer (containing 0.15 M sodium chloride), pH 4.5 (final volume 100 μL). The mixture was incubated at 37 ^{o}C for 24 hours.

The digestion products were separated by TLC using a pre-coated silica gel Kieselgel 60 (aluminium plate, 5×10cm, Merck, Darmstadt, Germany). Isopropanol: water (66:34) with 0.05 M sodium chloride was used for TLC development. The oligosaccharides were detected by spraying the plate with 0.5% (w/v) orcinol in 10% H_2SO_4 and heating at 120 ^{o}C.

2.6. BmHYA1 cloning and expression

2.6.1 Total RNA extraction

A pair of *Bm*K venom glands from a single scorpion was flash frozen in liquid nitrogen before being transferred to –70 ^{o}C until use. RNeasy mini Kit (Qiagen, Venlo, The Netherlands) was employed for the extraction of total RNA from the venom glands.

The experimental procedures were as follows:

1) The gland tissue with an estimated weight of 20 mg was taken out from –70 ^{o}C and put into liquid nitrogen. It was pulverized with clean mortar and pestle in liquid nitrogen. Liquid nitrogen was added from time to time during the operation

process to prevent it from drying out. Seven hundred microliters of buffer RLT (containing guanidine thiocyanate) with 0.1% β-mercaptoethanol was added into the mortar immediately after the liquid nitrogen was evaporated. The frozen sample blocks were then crushed with a pestle and the suspension was transferred with a Pasteur pipette into a clean 2 mL Eppendorf tube. The pulverized tissue was further homogenized in the tube by homogenizer.

2) The tube containing the sample was centrifuged at 16,000 $\times g$ for 3 minutes. The supernatant (~ 600 μL) was transferred to a new 1.5 mL tube and then thoroughly mixed by pipetting with 600 μL 70% ethanol (1:1). The mixture was applied successively (maximum 700 μL per time) to a RNeasy mini column by placing it in a 2 mL collection tube followed by centrifugation at 16,000 $\times g$ for 15 seconds. The flow-through was discarded.

3) Seven hundred microliters of buffer RW1 was added to the column subsequently and centrifuged at 16,000 $\times g$ for 15 seconds. The flow-through was discarded.

a. The column was transferred to a new collection tube, washed with 500 μL Buffer RPE by centrifuging at 16,000 $\times g$ for 15 seconds, and the flow-through was discarded. This step was repeated by centrifuging for 2 minutes.

b. The column was transferred to a new, clean 1.5 mL tube. Thirty microliters of RNase-free water was directly put onto the RNeasy silica-gel membrane in the column. After 1 minute incubation at room temperature, total RNA was released to the bottom of the tube by centrifuging the column at 16,000 $\times g$ for 1 minute.

4) Quality test of the RNA sample

Since the quality of the starting materials is critical for the molecular work, RNA sample was tested for its quality, including concentration, purity and integrity, before further processing.

i) Quantitation and purity determination

The concentration and the purity of the RNA sample were tested by Nanodrop (Thermo Scientific, Wilmington, DE, USA). RNA concentration was considered as 40 μg/mL when A260 value was 1. Sample with A260/280 ratio of ~ 2 was considered as a pure RNA. Two microliters of the sample was applied onto the machine and the result was automatically recorded.

ii) Integrity test

RNA sample is readily degraded by the RNase, which spreads broadly in animal tissues and the environment. Horizontal agarose gel electrophoresis was used to test

the integrity. RNA sample was run in 1% agarose gel for 40 minutes at 110 V using buffer TAE. Agarose gel was stained later by Golden SYBR staining solution for 30 minutes. The working solution was prepared freshly before use by diluting the SYBR Gold (5000X) solution that was stored in the dark. The band was detected under UV illuminator. Sample with clear 28S and 18S bands and without smear area under these two bands indicated the integrity of RNA sample.

2.6.2 First strand cDNA synthesis from Total RNA

First strand cDNA synthesis was accomplished by reverse transcription polymerase chain reaction (RT-PCR), using SuperScript™ II Reverse Transcriptase (SuperScript™ II RT) (Invitrogen, Carlsbad, CA, USA). Following this step, RNA template was degraded from cDNA:RNA hybrid molecule by RNase H.

Three microliters of total RNA (approximately 250 ng) was used to convert to cDNA. The procedures were according to the 3' RACE system manual provided along with the kit (Invitrogen, Carlsbad, CA, USA). Briefly, RNA was mixed with 1 μL of 10 μM Adapter Primer (AP, 5'-GGC CAC GCG TCG ACT AGT $AC(T)_{17}$-3'). AP takes the advantage of poly A+ tail of mRNA and contains extra nucleotide sequences to facilitate the later restriction enzyme-based molecular work. The mixture of RNA and AP was added with RNase free water to the final volume of 12 μL. The solution was incubated at 70 ^{o}C for 10 minutes and chilled on ice for 1 minute. Following components were then added to the reaction system:

10X PCR buffer	2 μL
25 mM $MgCl_2$	2 μL
10 mM dNTP mix	1 μL
0.1 M DTT	2 μL

The solution was equilibrated at 42 ^{o}C for 5 minutes before 1 μL SuperScript™ II RT was added. The system was kept at 42 ^{o}C for 50 minutes for the reaction, which was terminated by 15 minutes of incubation at 72 ^{o}C. One microliter of RNase H was added to the system (37 ^{o}C, 20 minutes) to degrade RNA template after the system was chilled on ice.

The product, i.e., single-stranded cDNA (ss cDNA), was stored at –20 ^{o}C for future amplification.

2.6.3 3’ rapid amplification of cDNA ends (3’-RACE)

3’ RACE is a technique to amplify the unknown DNA sequences using cDNA as a template and by taking the advantage of the natural poly A tail of the mRNA. When appropriate gene specific primer (GSP) at 5 prime and a universal amplification primer (UAP) was applied, the sequence in between would be amplified with a PCR program. In present work, the 3’ RACE experiment was conducted basically using the Invitrogen 3’RACE system (Invitrogen, Carlsbad, CA, USA).

2.6.3.1 Design of degenerate GSP

Degenerate primer can be designed based on a known amino acid sequence or

an amino acid sequence alignment. Amino acid sequence was reverse-translated to nucleotide sequence. Because of the degeneracy of the genetic code, in most cases it is impracticable to deduce the exactly nucleotide sequence from the known protein sequence. Then the primer would be a pool containing many possible sequences and thus the degenerate primer was actually a mixture. But too many primers mixed together would significantly reduce the specificity and even fail to prime the amplification. In addition, 3' end of the primer should be no degeneracy to avoid the unspecific extension. To balance up, the degenerate primer would be no more than 100 degeneracy and relatively short (17~23 bases) and no degeneracy at 3 prime (Pan et al., 2007). In this experiment, degenerate BmHAY1-GSP was designed based on the known segment of N-terminal amino acids of BmHYA1 (D-F-K-V-V-W-E), as 5'-GAY TTY AAR GTI GTN TGG GA-3', where I was Inosine, which was used for the substitution of all the four nucleotides A/C/T/G, therefore it reduces the degeneracy of the primer pool of 4-fold and N for A+C+T+G, Y for C+T, R for A+G, respectively. The primer was finally made as a degeneracy of 32, length of 20 bases and no degeneracy at 3' end. AUAP (5'- GGC CAC GCG TCG ACT AGT AC - 3') was provided by the commercial supplier. It contains the RE site sequence homologous to the adapter region of the AP. The fragment between these two segments was amplified, cloned and sequenced.

2.6.3.2 Amplification of 3' end cDNA of BmHYA1 with PCR

Polymerase chain reaction (PCR) was used for the 3' end amplification of

cDNA encoding BmHAY1 from *Bm*K venom ss cDNA library. The amplification was primed by the two oligonucleotides, BmHYA1-GSP and AUAP. BmHYA1-GSP was used as sense primer and AUAP for anti-sense.

The following components were added orderly into a clean PCR tube (50μL reaction system):

10X PCR buffer	5 μL
25 mM $MgCl_2$	3 μL
Autoclaved Milli Q water	36.5 μL
10 mM dNTP mix	1 μL
BmHAY1-GSP (100 μM solution)	1 μL
AUAP (10 μM)	1 μL
Taq DNA polymerase (5 units/μL)	0.5 μL
cDNA solution	2 μL

A PCR instrument was used to perform the typical PCR (Eppendorf, Mastercycler Gradient, Eppendorf Scientific, Westbury, NY, USA). To prevent evaporation, the lid temperature was set to 94 ^{o}C. Thirty four cycles were carried out beginning with 2 minutes of denaturing at 94 ^{o}C and ending by an extension step at 72 ^{o}C for 7 minutes.

The PCR program was performed as follows:

94 ^{o}C	2 minutes	1 cycle
94 ^{o}C	1 minute	
50 ^{o}C	1 minute	
72 ^{o}C	2.5 minutes	32 cycles
72 ^{o}C	7 minutes	1 cycle

The PCR program was performed with a relatively low annealing temperature of 50 ^{o}C. The degenerate primer was a mixture of various primers; the lower annealing temperature was set to facilitate the binding between primer and template. Together with this PCR, three other PCRs were carried out for control or alternative trial, which were primed by: rat GAPDH primer (5'-CCT TCA TTG ACC TCA ACT AC-3') and AUAP (as control), *Bm*KAS-1 primer (5'- GTC TGT TCA TTT GTG CTG ATA GGA G-3') and AUAP (as control), and hyaluronidase universal primer based on conserved region (SFHYA1-GSP, 5'- GSI GTI ATY GAY WKI GA-3', Ng et al., 2005) and AUAP (as alternative trial), respectively. All of the four PCRs were conducted simultaneously. After the reaction, five microliters of the PCR products of each reaction were applied to 1.5% agarose gel electrophoresis and stained by SYBR gold (Invitrogen, Carlsbad, CA, USA) for detection.

2.6.3.3 Agarose gel electrophoresis

Horizontal agarose gel electrophoresis is a widely-used technique to analyze DNA by their sizes. The fragments of DNA can migrate through agarose gel under an

electric field. DNA molecules, which bear negative charge, will migrate towards the positive electrode. DNA molecules of smaller size move faster than the larger ones. The result is visualized by SYBR Gold staining under UV light. In the experiment, the electrophoresis instrument was from Bio-Rad (Hercules, CA, USA), agarose gel powder was from Seakem LE (Bioproducts, Rockland, Maine, USA), 6X loading dye was from Promega (Madison, WI, USA), TAE buffer was supplied by the National University Medical Institutes, National University of Singapore. The experiment condition was 40 minutes at 110 V. Gels were stained with SYBR Gold for 30 minutes before being examined under UV light.

2.6.3.4 Isolation of DNA from agarose gel

DNA was isolated with QIA gel extraction kit (Qiagen, Venlo, Netherlands). The target DNA band was excised from agarose gel under UV illuminator with a clean scalpel and put into a clean 1.5 mL tube. Buffer QG was added into the tube (V_{QG}:V_{gel}=5:1) to dissolve the gel. The tube was incubated at room temperature until the gel was dissolved in the buffer (usually less than 1 hour). The solution was then applied into a QIA quick spin column, which was attached to a collection tube and centrifuged at 16,000 $\times g$ for 1 minute. The solution in the collection tube was discarded. The column was washed with 0.75 mL of buffer PE (with 75% ethanol) for 30 seconds and then placed into a new 1.5 mL tube. The bound DNA was eluted by addition of 30 μL elution buffer (provided in the kit) to the center of the spin column matrix followed by a centrifugation at 16,000 $\times g$ for 1 minute.

2.6.4 Enzymatic manipulation of DNA

All the restriction enzymes and buffers were from Promega (Madison, WI, USA). The enzymes involved were *Eco*R I, *Bam*H I, *Xho* I and T4 DNA ligase.

2.6.4.1 DNA ligation

pGEM-T Easy cloning kit (Promega, Madison, WI, USA) was employed for DNA ligation. In PCR, Taq polymerase may add a 3'-A overhang to both ends of the PCR product; the T-vector with 3'-T overhang at each end can connect to A and allow the fragment to ligate with the vector forming a recombinant plasmid. The connection was accomplished with T4 DNA ligase. A typical 10 μL ligation of cohesive ended TA cloning reaction system was composed as follows:

Vector DNA	1 μL (25 ng/mL)
Insert DNA	3 μL
2X ligase buffer	5 μL
T4 DNA ligase	1 μL (1 unit)

To obtain the maximum ligation efficiency, the reaction was carried out at 4 ^{o}C overnight and the reaction product was stored at -20 ^{o}C until use.

2.6.4.2 DNA digestion

The analytical restriction enzyme reaction was performed in a volume of 20 μL as follows:

Sterile, nuclease-free water	5 μL
Restriction enzyme 10X buffer	2 μL
BSA, Acetylated (1 mg/mL)	2 μL
DNA sample	10 μL
Restriction enzyme	1 μL

The reaction system was incubated overnight at 37 ^{o}C to achieve complete digestion.

2.6.4.3 Heat shock transformation and white/blue screening

i) Preparation of competent cells

The original DH5α competent cells were streaked on an antibiotic-free agar plate and incubated at 37 ^{o}C overnight. One single clone was taken and the cells grown at 37 ^{o}C with vigorous shaking to reach optical density (OD) 600 of 0.4-0.5 (mid log phase of cell growth, 1 cm path length). The cells were chilled on ice for 20 minutes before centrifugation (2,500 ×*g*, 15 minutes at 4 ^{o}C). The cell pellet was re-suspended with autoclaved ice-cold 0.1 M $CaCl_2$ (10 mL/50 mL of the culture). The cells were again centrifuged, 2,500 ×*g*, 15 minutes at 4 ^{o}C and the final cell pellet was re-suspended with ice-cold 10% glycerol in autoclaved 0.1 M $CaCl_2$ solution (2 mL/50 mL original culture). The DH5α competent cell suspension was aliquoted in

100 μL volumes and stored at -70 ^{o}C till use.

ii) Heat shock transformation of bacterial cells and white/blue screening

The stored DH5α was taken out from -70 ^{o}C and thawed on ice for 5 minutes. Five microliters of the ligation mixture was added to the cells and mixed by tapping the tube gently. The mixture was kept on ice for 20 minutes. The tube was then put in 42 ^{o}C water bath for exactly 45 seconds followed by immediate chilling on ice for 2 minutes. The cell suspension was transferred to a test tube with 400 μL of super optimal broth (SOC) medium and placed at 37 ^{o}C for 30 minutes to 1 hour with shaking. Meanwhile, 100 μL Isopropyl β-D-thiogalactopyranoside (IPTG, 100 mM) and 20 μL X-gal (40 mg/L) were spread onto an ampicillin-positive cultural plate. The cell suspension was subsequently plated evenly. The plate was then incubated overnight at 37 ^{o}C for the transformants to grow. This blue and white screening helps to detect the successful ligation in the transformants. The white clones were supposed to be the positive transformants. White colonies were picked up and transferred into 2 mL of lysogeny broth (LB) medium (with 50 μg/mL ampicillin) in a loosely capped 10 mL tube and incubated at 37 ^{o}C with vigorous shaking (220 rpm) for 14-16 hours.

2.6.4.4 Isolation of plasmids from the bacteria

To isolate plasmids from the bacteria, GE illustra™ plasmid Prep Mini Spin Kit (Piscataway, NJ, USA) was employed. The isolation was performed according to the manual, containing alkaline cell lysis and silica-based membrane binding and washing

procedures. Briefly, the bacteria suspension was harvested by centrifuge at high speed (16,000 ×*g*, 30 s). The supernatant was discarded and the bacteria pallet was re-suspended by isotonic solution containing RNase A (Solution I). Solution II (alkali buffer) was added subsequently to lyse the cells and the genomic DNA and proteins were denatured. The resultant solution was naturalized by Solution III, which is an acetate buffer and containing chaotropic salt. With centrifuge (16,000 ×*g*, 4 minutes), the unwanted components in the lysate, including genomic DNA, proteins and lipids, were precipitated. The supernatant containing plasmids was collected and transferred to column with a silica membrane. The membrane can retain the plasmid DNA. After a washing step, the plasmid DNA was eluted with an elution buffer.

2.6.4.5 Verification of the insert fragment

The verification of the insert is necessary before applying to DNA sequencing to avoid any false positive or non-specific insert. Two methods were used for this test.

i) Restriction enzyme digestion

The vector of pGEM contains restriction site of 5'-gaattc-3' for *Ecor*R I enzyme, which flank the cloning site. Digestion of *Ecor*R I would release the insert from the vector. *Ecor*R I enzyme package (Promega, Madison, WI, USA) was hence used for this test. The reactions were conducted as described in section 2.6.4.2. The plasmids containing the insert of expected sizes were sent for sequencing.

ii) PCR with T7 and SP6 primers

The vector of pGEM-T Easy contains T7 and SP6 promoter sequences at upstream and downstream respectively of the cloning site. After 14-16 hours of shaking, a small volume (0.5~1 μL) of LB medium which had considerable bacteria could be used as the template because it was supposed to contain the plasmid. The plasmid would be released at 94 ^{o}C and served as the template. With T7 and SP6 primers, the insert would be amplified by a PCR program. The PCR products would be examined by electrophoresis. The bacteria tubes which were tested to contain expected plasmids/inserts were proceed to plasmid isolation and DNA sequencing.

2.6.4.6 DNA sequencing

DNA sequencing was accomplished commercially, by 1st-Base Singapore. Plasmids (10 μL, 100 μg/mL) were sent for sequencing directly. Primers M13 Forward/Reverse, T7 promoter, S • tag coding sequence, T7 terminator and specific internal primers were chosen for different DNA samples.

2.6.5 Protein sequence analyzing

The mature protein was deduced by the nucleotide sequence. A number of hyaluronidases were aligned by Cluster W method (Thompson et al., 1994), using Lasergene software (DNASTAR, Madison, WI, USA). The sequence comparison included 9 hyaluronidases: BmHYA1 (present work), honeybee venom HYA (honeybee venom hyaluronidase, AAA27730.1), SFHYA1 (stonefish venom

hyaluronidase, AAO74499.1), Snake Eo HYA (snake *E. ocellatus* venom hyaluronidase, ABI33937.1), HYAL1~4 (human hyaluronidases 1~4, CAG46731.1, NP_149348.2, NP_003540.2, NP_036401.2), Human PH-20 (AAC60607.2). To determine diversification of the hyaluronidase enzymes, phylogenetic analysis was employed. A neighbor-joining method was used to construct the phylogenetic tree implemented by MegAlign, in Lasergene software (DNASTAR, Madison, WI, USA). Twenty-seven hyaluronidases were selected for phylogenetic analysis, including both venom and non-venom hyaluronidases, they are: BmHYA1 (present work), *C. elegans* hyaluronidase (CAA88874.1), sand fly hyaluronidase (AAD32195.1), honeybee (*Apis mellifera*) venom hyaluronidase (AAA27730.1), paper wasp hyaluronidase (AAD52616.1), white-face hornet hyaluronidase (AAA68279.1), yellow jacket wasp hyaluronidase (AAB48073.1), solitary wasp *A. samariensis* (BAF93867.1), mouse serum hyaluronidase (AAC15949.1), human HYAL-1~4 (CAG46731.1, NP_149348.2, NP_003540.2, NP_036401.2), snake *C. cerastes* venom hyaluronidase (ABI33940.1), snake *E. ocellatus* venom hyaluronidase (ABI33937.1), snake *B.arietans* venom hyaluronidase (ABI33945.1), rat hyaluronidase (AAD01980.1), mouse HYAL2 (AAK28481.1), SFHYA1 (stone fish venom hyaluronidase, AY232496), xenopus kidney hyaluronidase (AAD28281.1), rat sperm surface antigen, 2B1 (CAA62016.1), red fox PH-20 (AAB86588.1), cynomolgus monkey *M.fascicularis* PH-20 (L13780), mouse PH-20 (BAC55070.1), human PH-20 (AAC60607.2), rabbit PH-20 (AAA88913.1) and Guinea pig PH-20 (CAA39768.1).

2.6.6 Expression of BmHYA1

The expression of BmHYA1 was conducted using *E.coli* expression system, taking its advantage of being able to provide adequate amount of recombinant proteins. Both GST and His tag purification methods were employed. The sense primers for GST and His tag purification were GCC GGA TCC ACA TCT GCC GAC TTT AAG GTG and GAT CAT ATG CAT CAC CAT CAC CAT CAC ACA TCT GCC GAC, containing *BamH* I and *Nde* I restriction sites, respectively. The antisense primer was ATT GTC AAA TTG CAC CTT GAC TCG AGC GCG for both GST and His tag expressions, containing *Xho* I restriction site. The sequence ACA TCT GCC in sense primers were artificially coded based on the Edman degradation results, for the first 3 N-terminal amino acid residues: T-S-A. Since in the cloning work, 20 clones (named F1 to F20) were screened and 10 were identified to contain the BmHYA1 3' fragment insert. A positive Clone F18 was randomly selected among those clones which contain the *standard* BmHYA1 cDNA 3' end sequence (for "standard sequence", see section 3.12.4), as the PCR template. The PCR products, i.e., the cDNA fragments of interest were subcloned at *Bam*H I and *Xho* I, or *Nde* I and *Xho* I, sites of the pET41a(+) vector (Novagen, San Diego, CA, USA) and amplified with DH5α competent cells. The insert was confirmed in the correct reading frame by DNA sequencing. BL21 competent cells (Novagen, San Diego, CA, USA) were transformed by chimeric plasmids with GST or His tags. The transformants were cultured on the LB plate (with 50 μg/mL kanamycin). A single clone was selected to culture in 5 mL LB medium (with 50 μg/mL kanamycin), shaking at 220 rpm, 37 ^{o}C

overnight. Then 100 mL LB medium (with 50 μg/mL kanamycin) was inoculated with 2 mL LB medium, which was taken out from the above 5 mL LB medium. The bacteria were incubated at 37 ^{o}C with agitation until its OD value achieving 0.4-0.8 (about 2 hours). IPTG was then added to the cultural medium to reach a final concentration of 0.4 mM. The medium was collected after 4 hours of IPTG addition. Before IPTG was added, 3 mL of the cultural medium was collected as the expression control. The expression of recombinant protein collected, concentrated and purified by GST purification or His purification methods, according to the product manuals respectively (GST: GST purification modules, GE healthcare, Piscataway, NJ, USA; His: Protino, Macherey-Nagel, Düren, Germany). The existence of the expressed recombinant BmHYA1 was examined by SDS-PAGE. The activity of recombinant BmHYA1 was tested by the method described in section 2.3.

2.7 Biological activity

2.7.1 Cell culture

The human breast cancer cell line MDA-MB-231 was a gift from Dr. Bay B.H., Dept. of Anatomy, National University of Singapore. This cell line is selected due to its availability and its aggressive property. In pilot study, MCF-7, a non-invasive breast cancer cell line and MDA-MB-231, a highly metastatic breast cancer cell line (Tai et al., 2003) were stained and compared, MDA-MB-231 was confirmed to contain much more hyaluronan and chosen as the experimental subject. The cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM, ATCC, Manassas, VA,

USA), supplemented with 10% fetal bovine serum (FBS, Gibco-Invitrogen, Carlsbad, CA, USA), at 37 ^{o}C, under 5% CO_2 in a humid atmosphere.

2.7.2 Immunohistochemical staining for hyaluronan

MDA-MB-231 cells were sub-cultured in 96-well plate, in the presence and absence of BmHYA1 (approximately 100 units) for 20 hours. The cells were fixed with 4% paraformalclehyde for 30 minutes. After washing 3 times with PBS, the cells were pre-incubated with 6% normal goat serum (NGS) in PBS-T (PBS with 0.2% Tween) for 60 minutes at room temperature and washed with PBS for 3 times. The cells were then incubated with 200 μL of diluted biotinylated HA-binding peptide (bHABP, Sigma, St. Louis, MO, USA) at the final concentration of 1.25 μg/mL, overnight at 4 ^{o}C. Finally, the wells were washed for 3 times with PBS and incubated for 1 hour with avidin-labeled horseradish peroxidase solution (Pierce, Rockford, IL, USA). Quantitative analysis of 3,3'-diaminobenzidine (DAB) staining was done by measuring the OD values of the cells (using ImageJ software, U. S. National Institutes of Health, Bethesda, Maryland, USA) against the background.

2.7.3 Western blot analysis for investigating the effect of the enzyme on the expression of cancer-related biological molecules

CD44 was chosen as the study subject is because it is one of the main receptors of hyaluronan (Stern et al., 2001). CD44 has various isoforms (Naor et al., 1997); in pilot study, CD44 standard isoform and variant isoforms 7 and 6 (CD44v6) were

tested for the possible expression changes by treatment of hyaluronidase and the first two isoforms were not detected to have obvious fluctuation, hence CD44v6 was selected for the further study. In the test, cells were incubated in the medium in the presence and absence of BmHYA1 (approximately 100 units), for 12, 24 and 48 hours. Cells were lysed with cell lysis buffer (Pierce, Rockford, IL, USA) and centrifuged at 12,000 $\times g$ to remove the insoluble platelets. Protein concentrations were determined using Bio-Rad protein assay kit (Hercules, CA, USA). The calibrated protein samples were loaded onto 8% sodium dodecyl sulfate-polyacrylamide gel for electrophoresis and then transferred to nitrocellulose membranes. The membranes were blocked in PBS-T (PBS with 0.2% Tween-20) solution with 5% skim milk for 1 hour. After that the membranes were incubated with 1:200 dilution of anti-CD44v6 monoclonal antibody (Santa Cruz, Santa Cruz, CA, USA) or 1:5000 anti-β actin mAb (Santa Cruz, Santa Cruz, CA, USA) in PBS-T overnight at 4 ^{o}C. The membranes were washed with PBS-T for 15 minutes three times and then incubated for 45 minutes with horseradish peroxidase-conjugated anti-mouse IgG Ab (Bio-Rad, Hercules, CA, USA) for CD44v6 and β-actin mAbs. The secondary antibody was detected by chemiluminescence system (Amersham, Piscataway, NJ, USA).

2.8 **Statistical analysis**

The quantitative data were obtained in triplicate experiments and were expressed as mean ± S.E.M. The difference between any two groups was examined by student's *t*-test. Significance was considered at $p<0.05$.

CHAPTER III

RESULTS AND OBSERVATIONS

3.1 The crude venom

After dissolution, centrifugation and filtration, measurement of protein concentration of the crude venom solution by Bio-Rad protein assay indicated that protein accounted for 36% of the total weight of the crude venom.

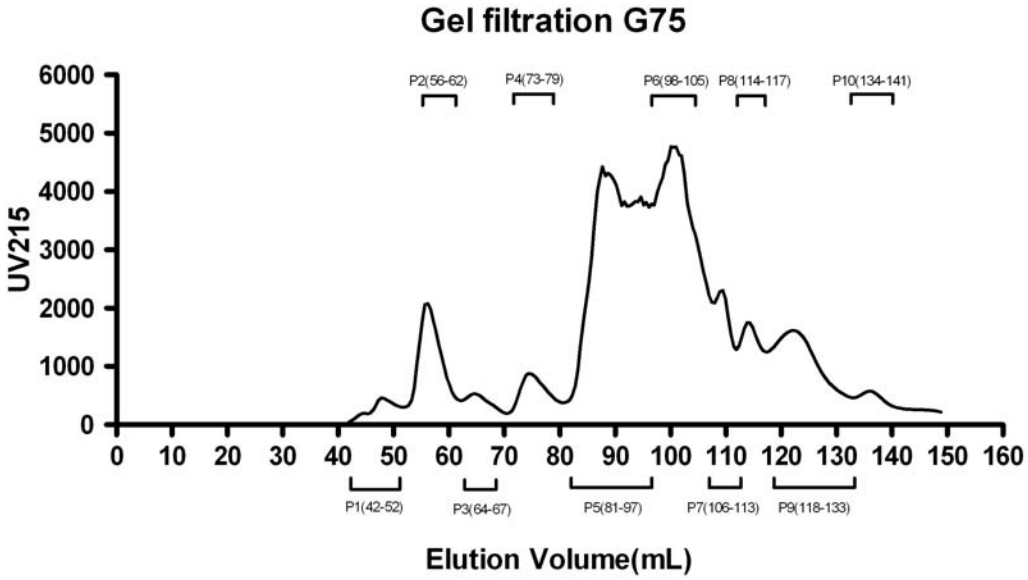
3.2 Preliminary separation of BCV and biological activity screening

3.2.1 Preliminary separation of BCV

In the first step, BCV was separated by gel filtration on Superdex G75 column. A total of 10 peaks eluted from the column were collected and pooled to represent different fractions: peak 1 (42~52 mL), peak 2 (56~62 mL), peak 3 (64~67 mL), peak 4 (73~79 mL), peak 5 (81~97 mL), peak 6 (98~105 mL), peak 7 (106~113 mL), peak 8 (114~117 mL), peak 9 (118~133 mL) and peak 10 (134~141 mL). The numbers in parentheses showed the elution volumes (as indicated by horizontal bars in Fig.3.1.(A). The components of 10 peaks were pooled and subjected to 15% SDS-PAGE to analyze the M.W. distributions. The M.W. distribution pattern of SDS-PAGE was found to be consistent with the chromatographic behavior of the eluted fractions, indicating gradual distribution of proteins from high to low M.W. species (Fig. 3.1.B). To further investigate the detailed distribution pattern of high M.W. components, aliquots of eluants from every alternate tubes were subjected to 15% SDS-PAGE. SDS-PAGE analysis (Fig. 3.2) confirmed that in BCV, there was a preponderance of high M.W. proteins having M.W. greater than 14 kDa, with the highest found to be

around 200 kDa, while the majority of proteins had M.W.s between 14 and 75 kDa. The high M.W proteins were mainly eluted in peaks 1 ~ 4, after which the eluates in later fractions contained mainly small peptides with M.W.s below 10 kDa. Since common SDS-PAGE cannot separate very low M.W. peptides, MS was used instead to investigate the M.W. distributions of the collected eluates from peaks 5 to 10. Based on the MS results, the low M. W. peptides can basically be classified into two groups, one with a M.W. around 3 kDa and the other around 7 kDa. These results are in agreement with those reported previously for *Bm*K scorpion venoms (see review Goudet et al., 2002). When checked for the M. W. distribution pattern of the eluted proteins in individual peaks, it was found that the peptides in peaks 5 and 7 had M.W.s around 3 kDa, while peak 6 contained peptides with M.W. evenly distributed between 5~8 kDa. Peak 8 consisted of about 7 kDa peptides, whereas peak 9 contained both of the two groups, and lastly peak 10 had the composition similar to peak 9. The protein concentration of the pooled elution peaks were then measured preparing for screening of biological activities.

(A)



(B)

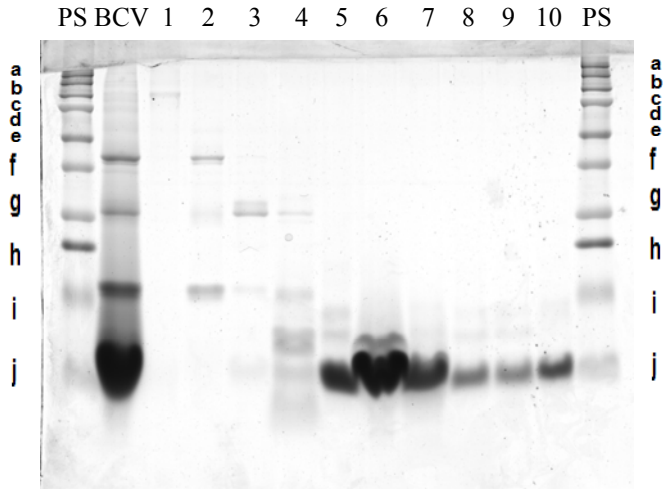


Fig.3.1. Gel filtration chromatography (A), and SDS-PAGE pattern of the gel filtration factions (B).
(A): Crude venom was separated by gel filtration on Superdex G75 column. The elution buffer was 50mM NH_4HCO_3, pH 8.9. The fractions representing 10 peaks were pooled as indicated by the horizontal bars. (B): 15% SDS-PAGE profile for BCV and 10 pooled peaks. Lanes from left to right: PS (Protein standards), BCV, peaks 1 to 10, PS. The protein standards in lanes 1 and 13 (shown in duplicate) indicated the M.W. (kDa) of (a)250, (b)150, (c)100, (d)75, (e)50, (f)37, (g)25, (h)20, (i)15, and (j)10, respectively.

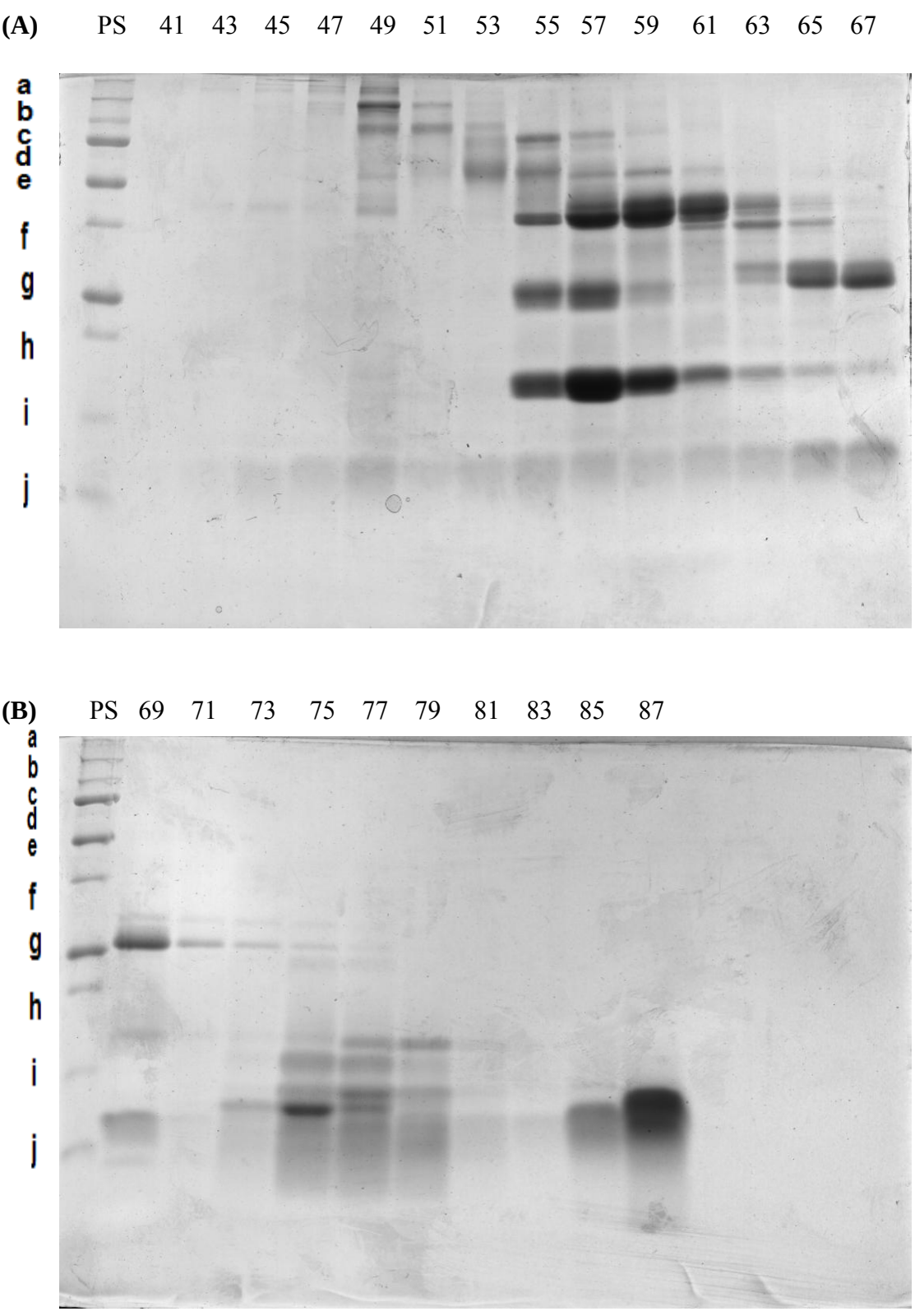


Fig. 3.2. SDS-PAGE profile of gel filtration fractions eluted from peak 1 to 4. Eluates from alternate tubes covering elution volumes from 41 to 87 mL were examined. (A) From left to right, PS (protein standards), tubes 41~67 (mL). (B) From left to right, PS, tubes 69~87 (mL). Protein standards (kDa): (a)250, (b)150, (c)100, (d)75, (e)50, (f)37, (g)25, (h)20, (i)15 and (j)10.

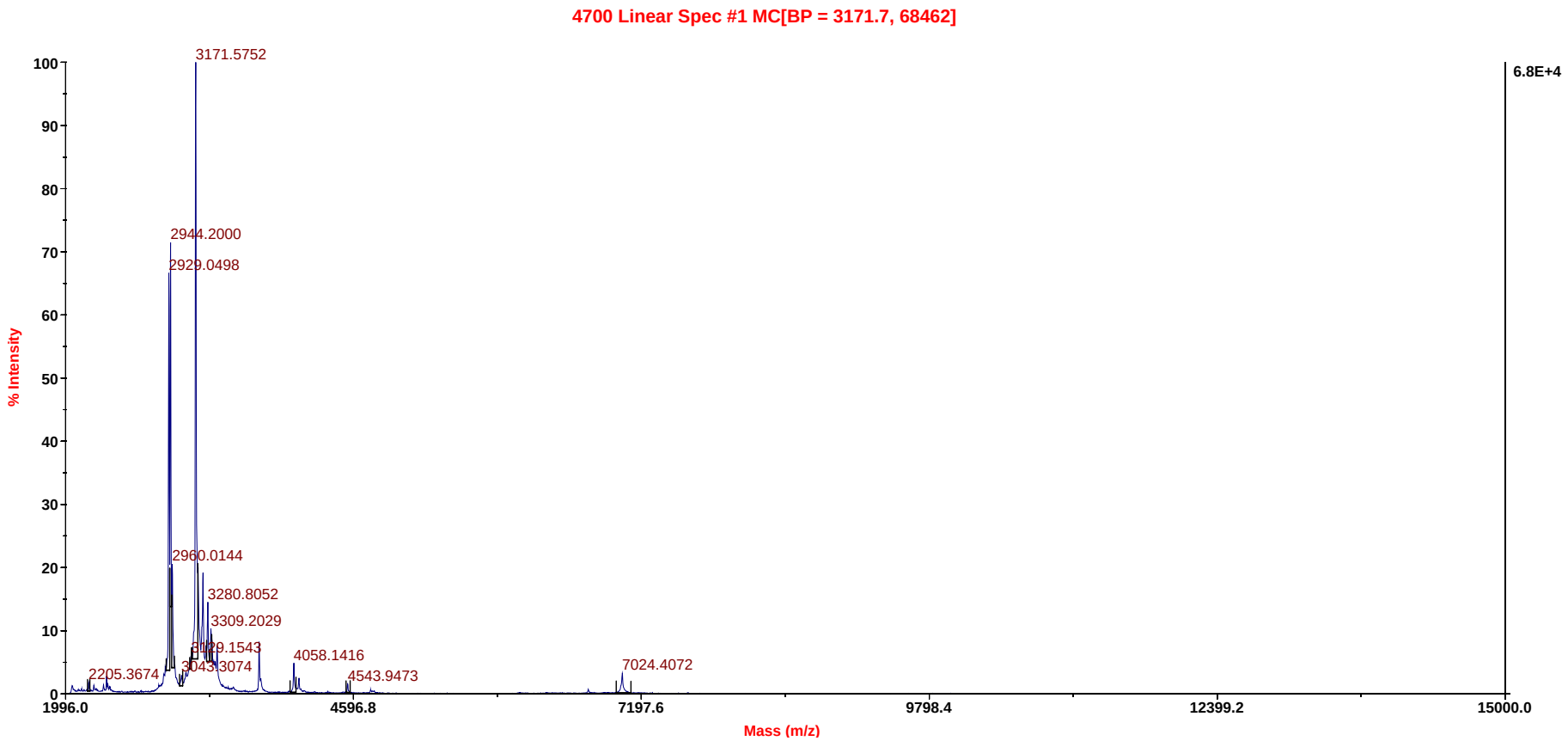


Fig. 3.3. Mass spectrum of peak 5 from gel filtration chromatography.

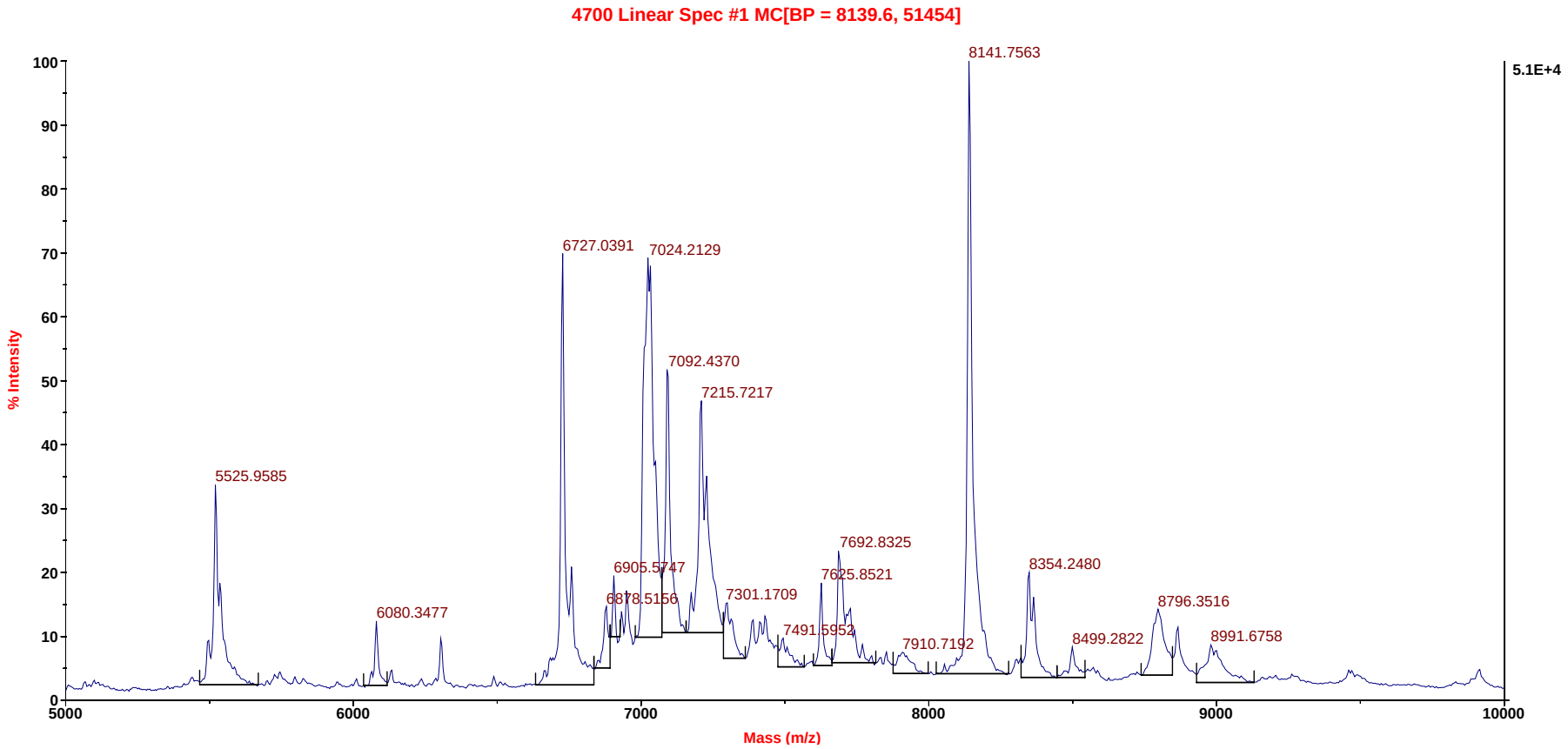


Fig. 3.4. Mass spectrum of peak 6 from gel filtration chromatography.

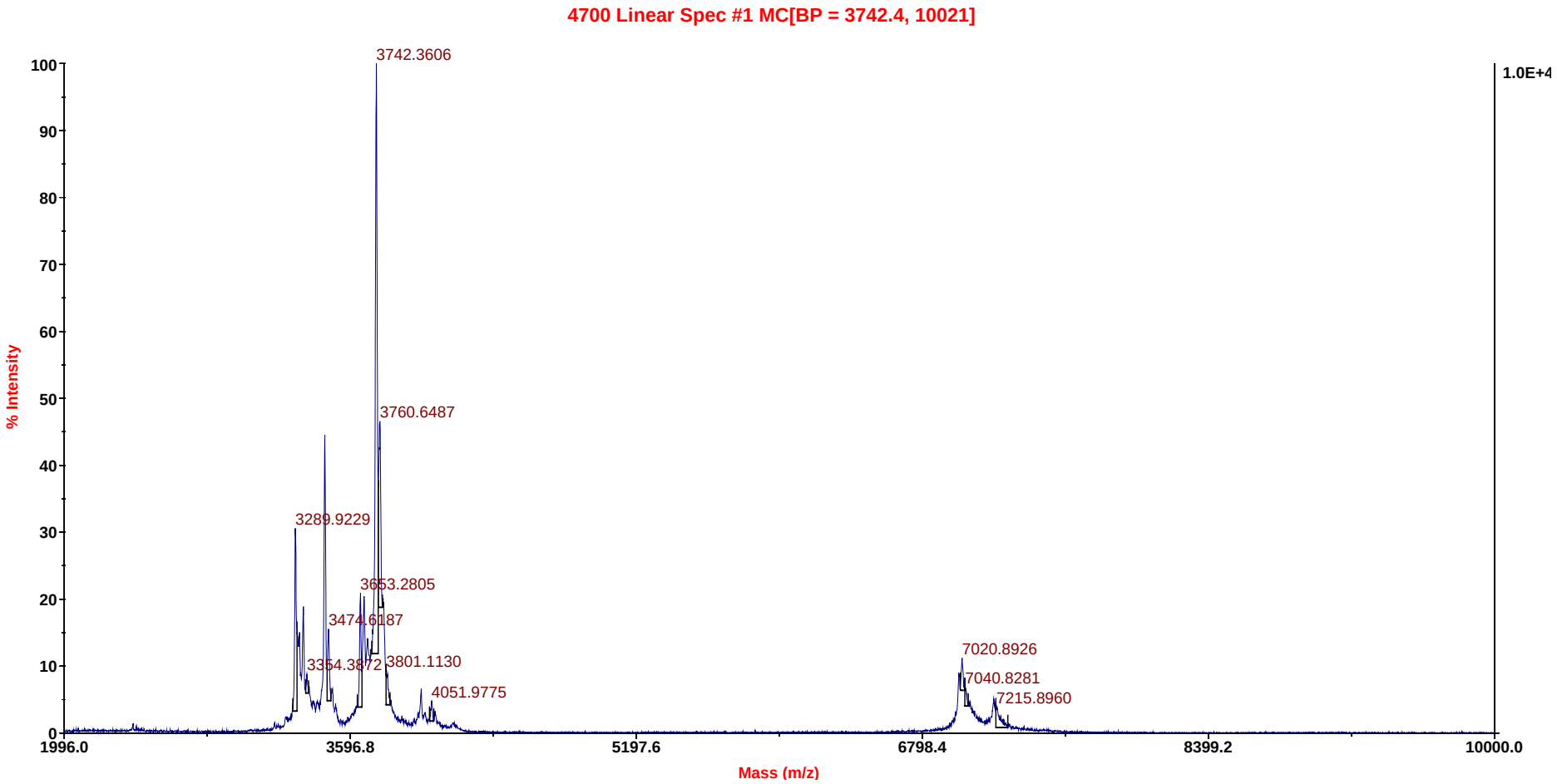


Fig. 3.5. Mass spectrum of peak 7 from gel filtration chromatography.

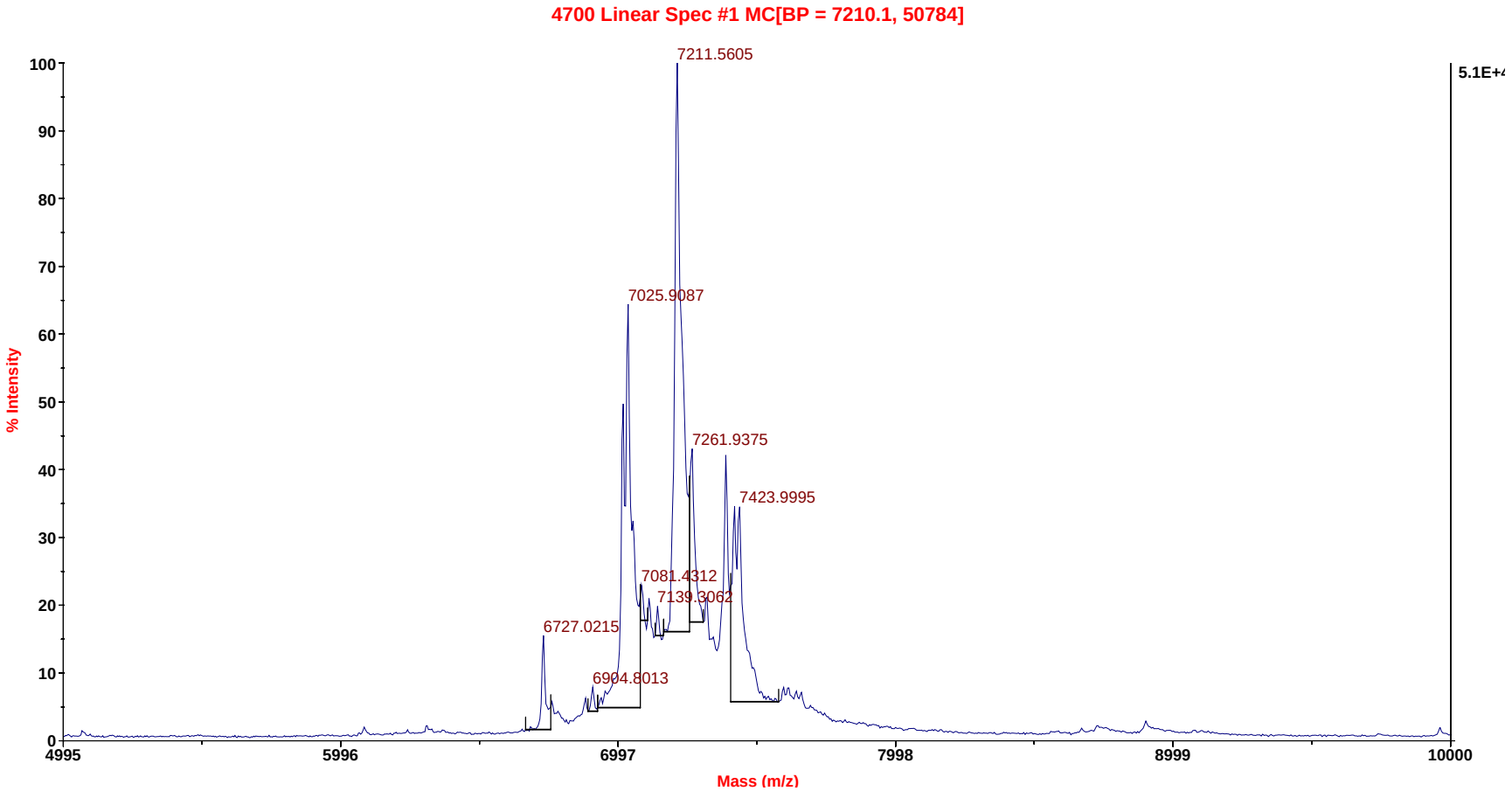


Fig. 3.6. Mass spectrum of peak 8 from gel filtration chromatography.

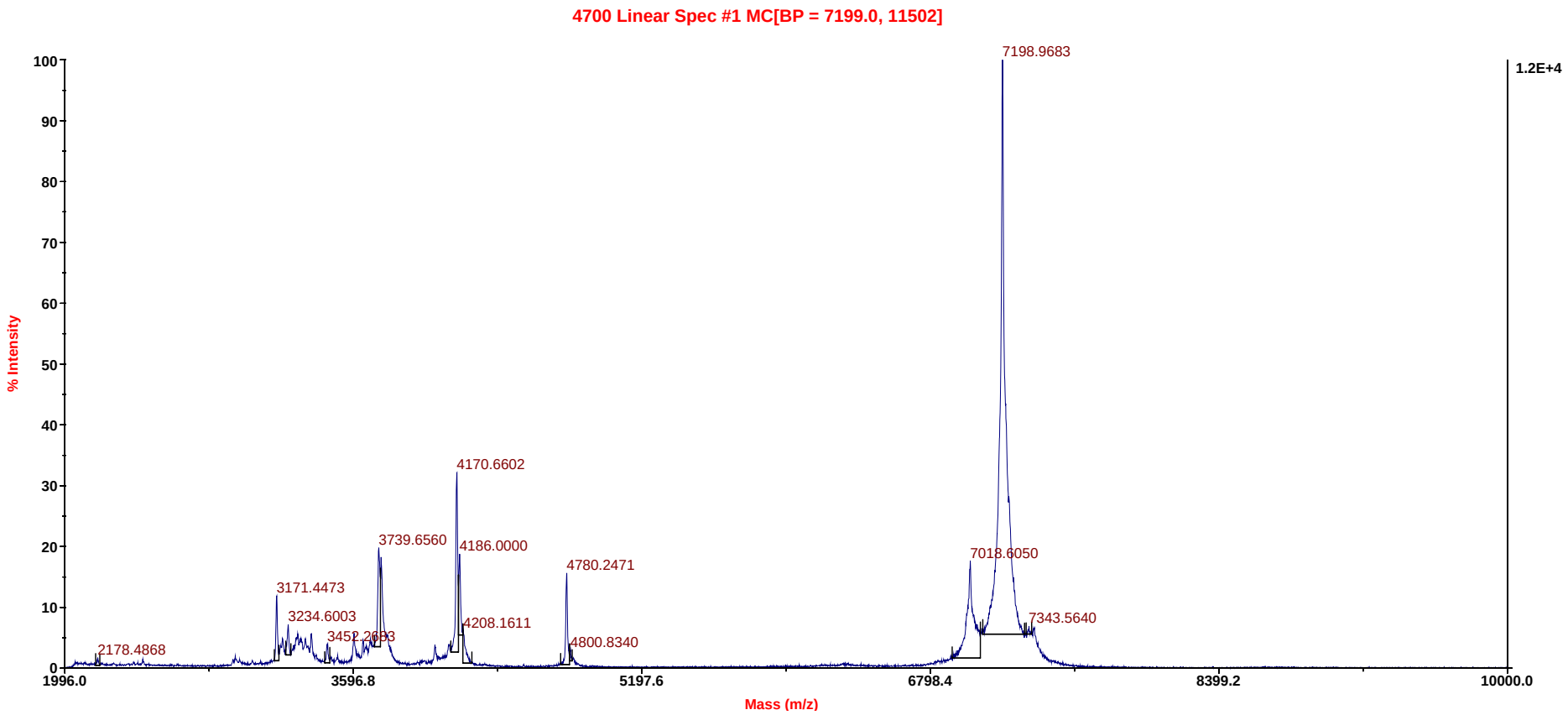


Fig. 3.7. Mass spectrum of peak 9 from gel filtration chromatography.

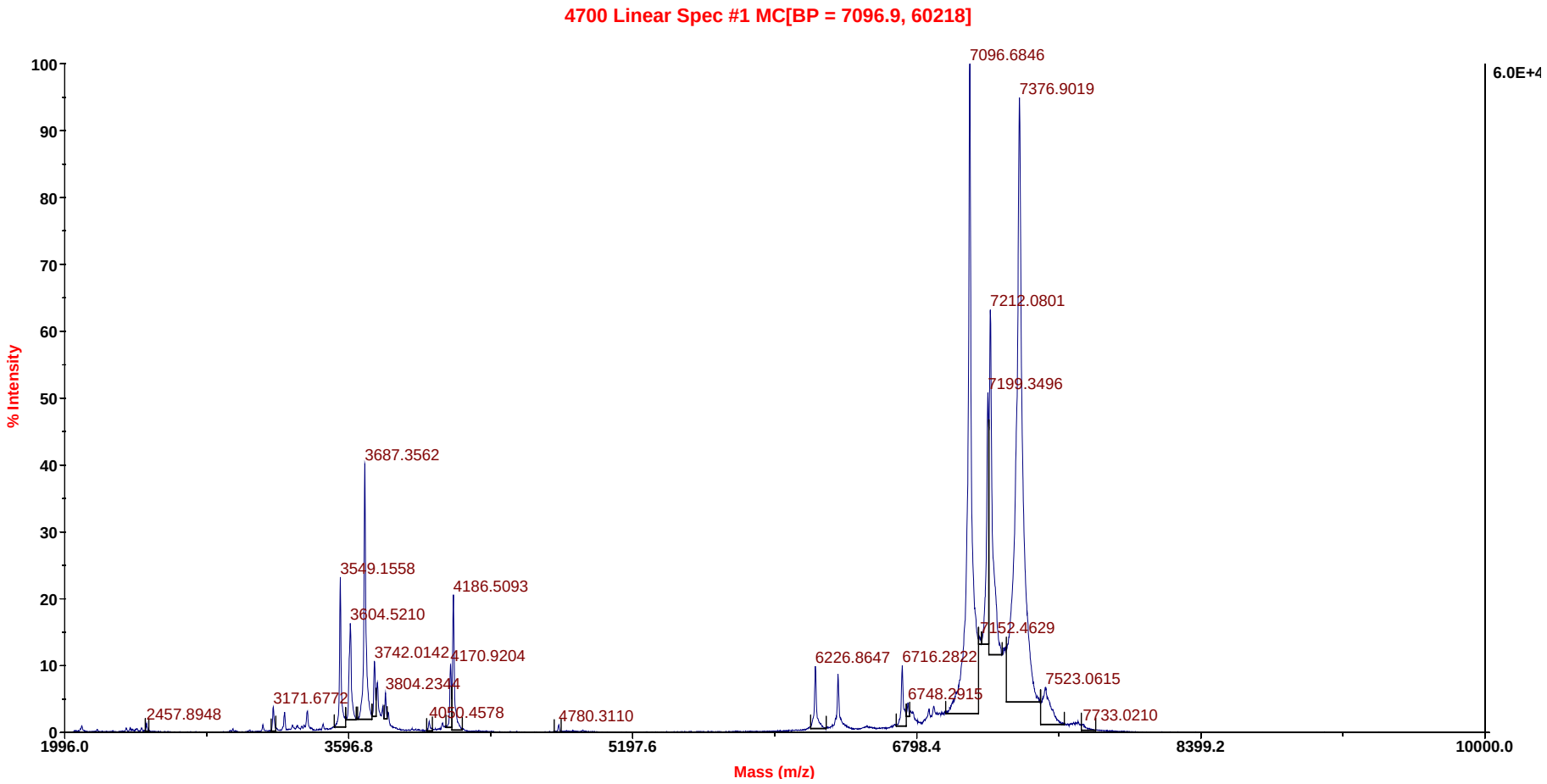


Fig. 3.8. Mass spectrum of peak 10 from gel filtration chromatography.

3.2.2 L-amino acid oxidase (LAAO)activity:

LAAO enzyme activity was identified in BCV. The activity was much weaker compared to that of snake *Naja Kaouthia* crude venom (Fig. 3.9), which was included as a positive control in the experiment.

3.2.3 Fibrinogenolytic activity

The activity was mainly associated with the first 3 peaks based on SDS-PAGE analysis (Fig.3.10). The Aα-chain of fibrinogen was apparently degraded in the first three peaks (indicated by a red circle), while the Bβ- and γ chains were barely affected. Further experiment was conducted to narrow down the active fractions. Every alternate tubes collected after chromatography (i.e., elution volume from 47 to 81 mL) were monitored for the activity. The absence of bands in the SDS-PAGE of the fraction corresponding to tubes #55 to 57 indicated that Aα chains were degraded, whereas the other tested fractions remained intact indicating that no degradation was apparent. The venom was thus confirmed to have Aα fibrinogenolytic activity.

3.2.4 Hemolytic activity

The effect on both the whole blood (Fig. 3.11) and the red blood cell (RBC) (Fig.3.12) were tested. The hemolytic activity was found in all the fractions but not in crude venom. The highest effect was found in Peak 7. The negative control was the NH_4CO_3 buffer, and the positive control was Triton X-100. The activity of all the 10 fractions showed relatively weak effect on both whole blood and RBC as compared to

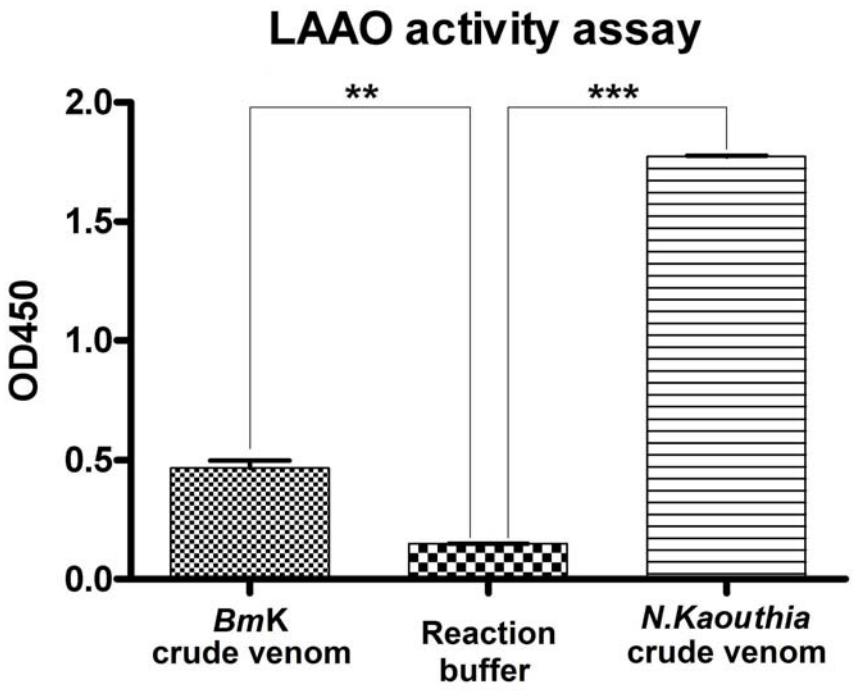
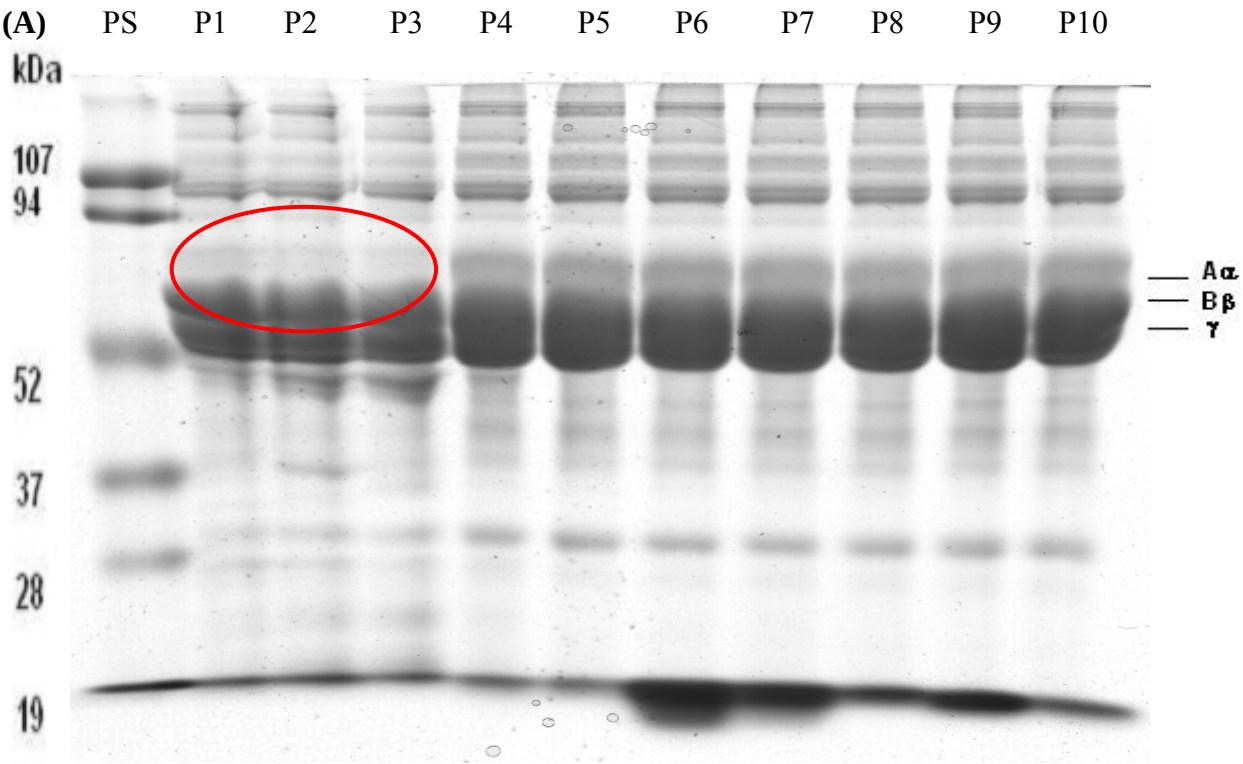
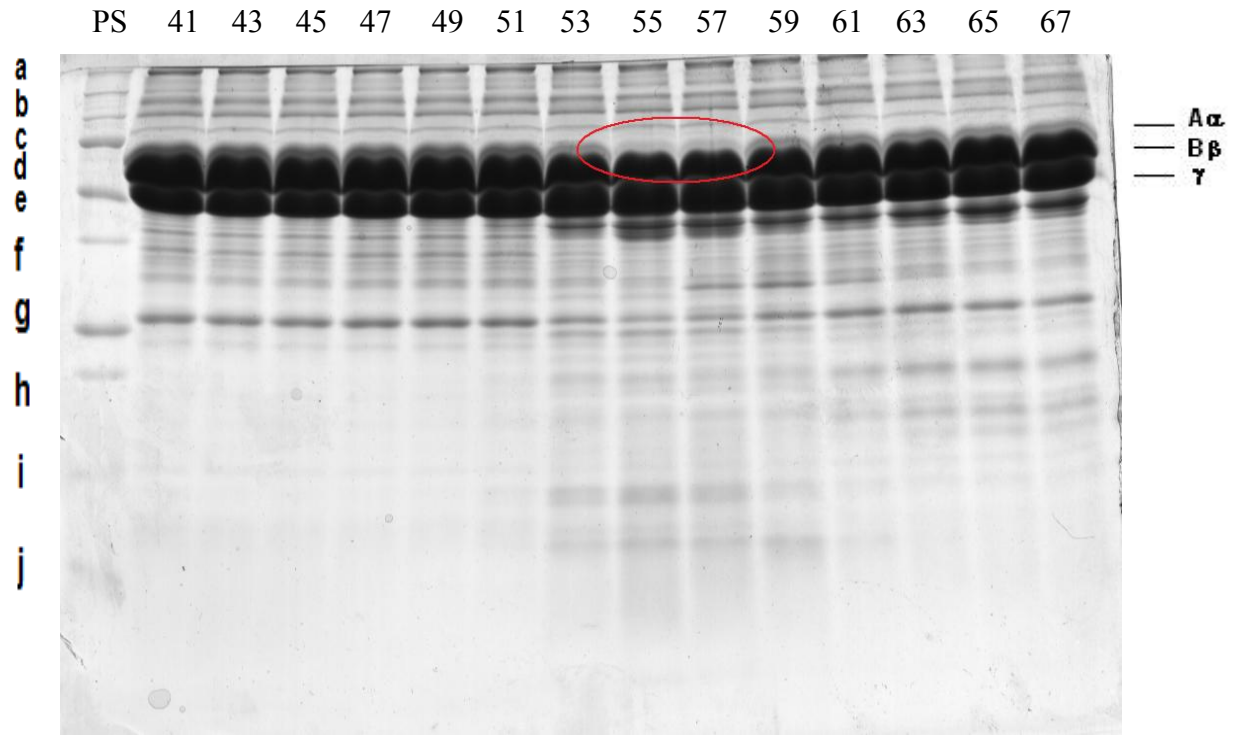


Fig.3.9. LAAO activity test. It showed that BCV contained LAAO activity, but the activity of BCV was much weaker than that of snake *Naja Kaouthia* crude venom. Compared to negative control: **: $p<0.01$; ***: $p<0.001$.


(A)
PS
P1
P2
P3
P4
P5
P6
P7
P8
P9
P10
kDa
107
94
52
37
28
19
Aα
Bβ
γ


(B)
PS
41
43
45
47
49
51
53
55
57
59
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(C)

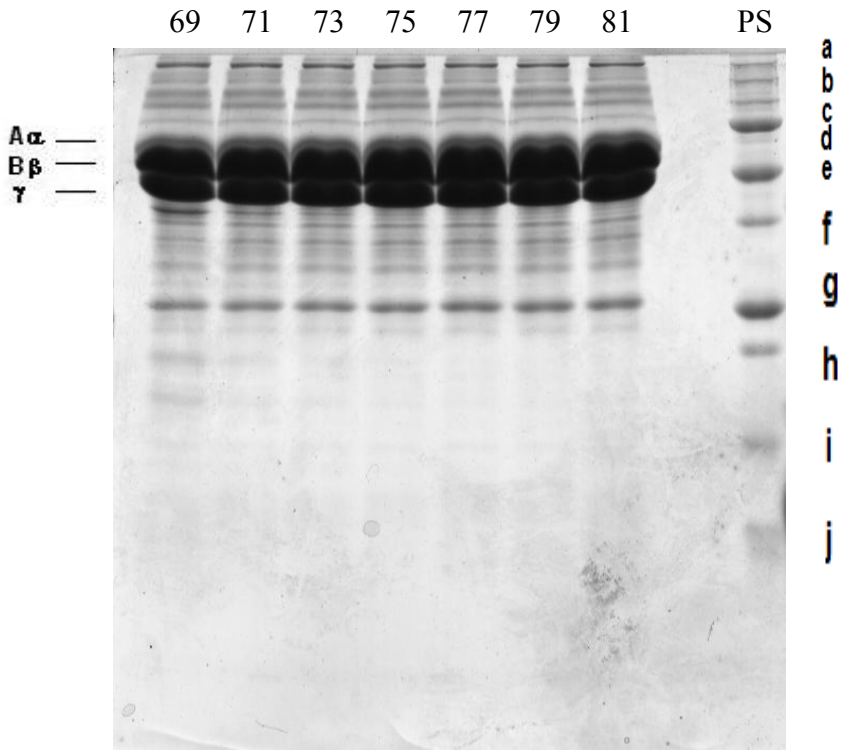

Fig.3.10. Fibrinogen degradation in the gel filtration factions.
(A), pooled fractions representing the 10 peaks. Lanes from left to right: PS (protein standards), followed by fibrinogen bands P1~P10, representing peaks 1~10. Note that the fibrinogen Aα chains were degraded (bands absent) in P1, P2 and P3 (marked by red circle); (B~C), alternate fractions collected throughout the chromatographic step.
(B), Lanes from left to right: PS (protein standards), followed by fibrinogen bands from the alternate fractions (tubes #41~67). Note that fibrinogen Aα chains were degraded in lanes 9~10 (tubes #55 and #57). (C), Lanes from left to right: fibrinogen bands from alternate fractions (tubes #69~81), and PS (last lane). The results confirmed that the venom exhibited Aα fibrinogenolytic activity.
For protein standards (kDa): (A) directly shown in figure; (B) and (C): (a)250, (b)150, (c)100, (d)75, (e)50, (f)37, (g)25, (h)20, (i)15 and (j)10.

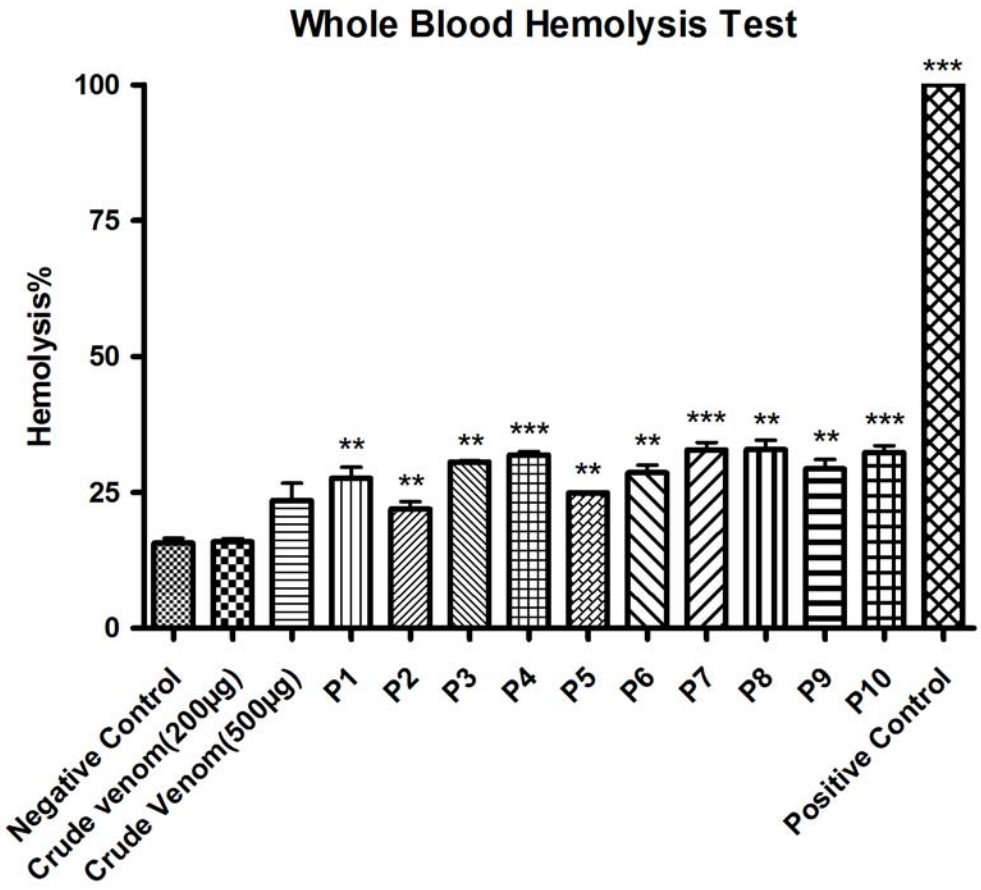

Fig. 3.11. Whole blood hemolytic tests for BCV and all fractions. All the 10 fractions showed hemolytic activity on rat whole blood, whereas BCV did not. Asterisks indicated the significant difference between individual treatment group and control group, respectively. Compared to negative control: **: $p<0.01$; ***: $p<0.001$.

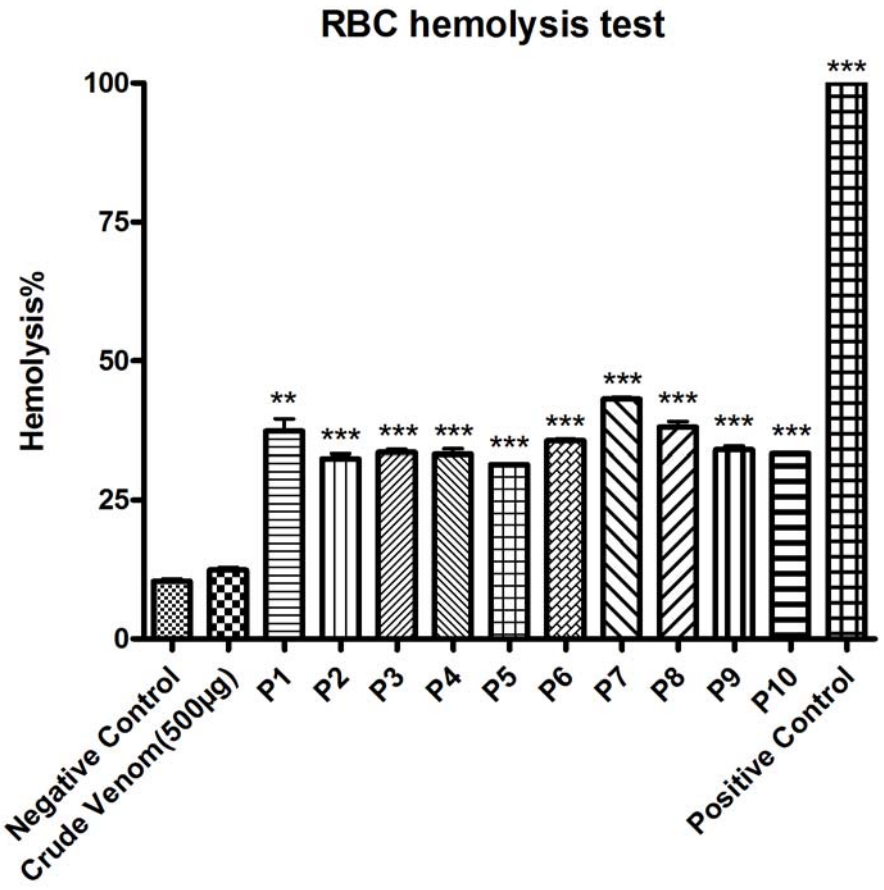

Fig. 3.12. Red blood cell tests for the BCV and all the 10 fractions. All the fractions, but not the crude venom, showed hemolytic activity on RBC. Asterisks indicated the significant difference between individual treatment group and control group, respectively. Compared to negative control: **: $p<0.01$; ***: $p<0.001$.

the positive control.

3.2.5 Antibacterial activity

Neither the BCV (used at 1000 μg) nor all of its fractions showed any antibacterial activity. The crude venom from *Naja Kaouthia* snake used as a positive control showed obvious antibacterial activity at 100 μg. A round bacteria growth inhibition zone was observed where *N. Kaouthia* venom was applied (Fig.3.13, indicated by an arrow), while no other bacteria growth inhibition zone was found on the agar.

3.2.6 Amidolytic activity

Amidolytic activity was detected in *Bm*K venom. As shown in Fig 3.14, the eluate fraction (i.e., 50~65 mL of elution volume) was observed to directly hydrolyze S-2238. The activity region covered almost all the high M.W. protein fractions.

3.2.7 PLA_2 activity

PLA_2 activity was detected in the high M.W. eluates. Two activity peaks were located in peak 1, and also in between peaks 2 and 3 (Fig. 3.15). This indicated that *Bm*K venom may have at least two high M.W. PLA_2 isoforms.

3.2.8 Hyaluronidase activity

The hyaluronidase activity was concentrated in peak 2. The activity of hyaluronidase at each chromatographic step was described in detail in the following

section.

The results of the screening were summarized in Table 3.1.

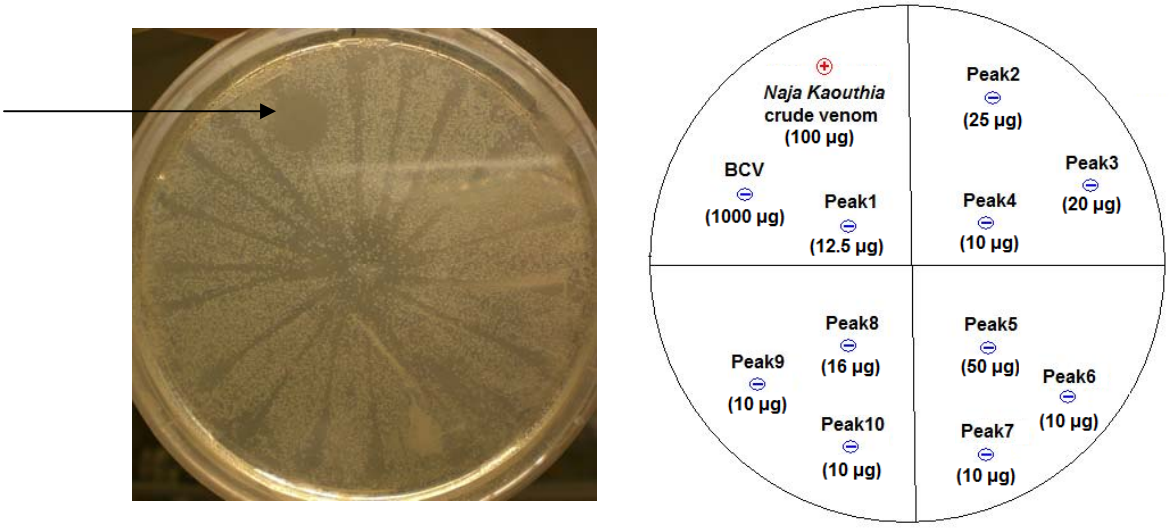

Fig.3.13. Test for anti-bacterial activity. The sketch map indicates the loading positions (approximately at blue and red small circles) of the samples. *Naja Kaouthia* crude venom (positive control, indicated by arrow in left figure) showed strong anti-bacterial activity. For *Bm*K, neither crude venom nor its fractions showed any anti-bacterial activity.

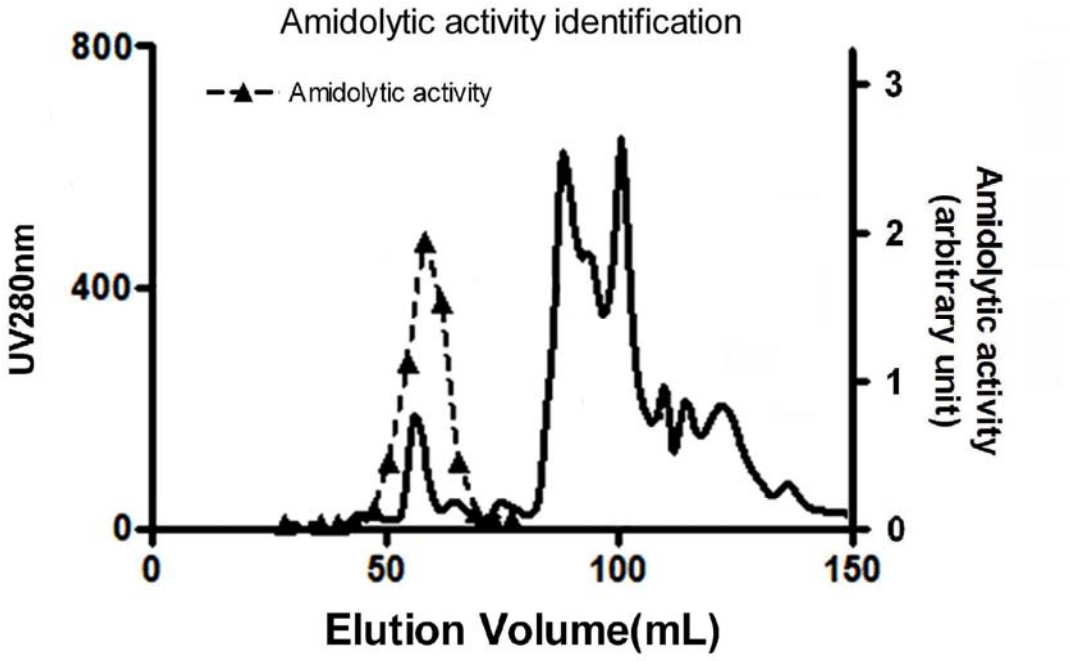

Fig. 3.14. Amidolytic activity identification with Gel filtration superdex G75 chromatography. The fractions with amidolytic activity on S-2238 were indicated by (**-▲-**). The activity covered over almost the whole high M.W. region of the chromatogram.

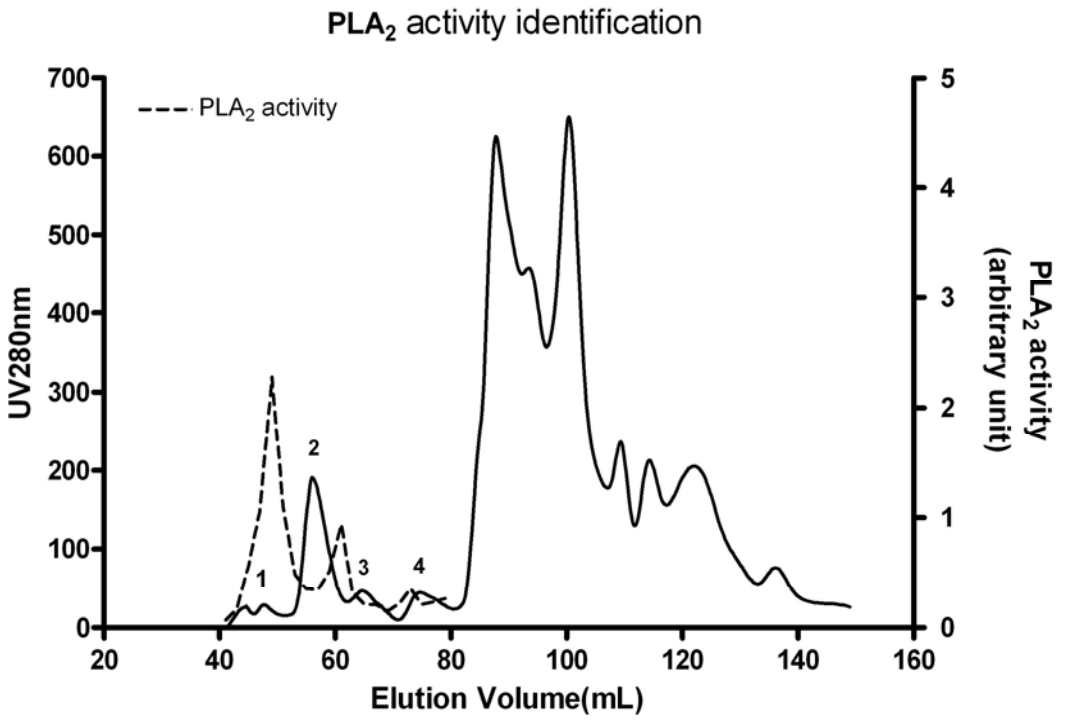

Fig. 3.15. PLA_2 activity identification with Gel filtration superdex G75 chromatography. The fractions with PLA_2 activity were indicated by the dash-dotted line. It showed two activity peaks in 1), peak 1 (around fraction #49) and 2), in between peaks 2 & 3 (around fraction #61).

Table 3.1. The results of screening of *Bm*K venom activity.

Activities		Crude venom	Peak1	Peak2	Peak3	Peak4	Peak5	Peak6	Peak7	Peak8	Peak9	Peak10
LAAO		+	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Fibrinogenolytic		N.D.	+	+	+	-	-	-	-	-	-	-
Hemo-Lysis	RBC	-	+	+	+	+	+	+	+	+	+	+
	Whole	-	+	+	+	+	+	+	+	+	+	+
Anti-bacterial		-	-	-	-	-	-	-	-	-	-	-
Amidolytic		N.D.	+	+	+	-	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
PLA_2		N.D.	+	+/-	+/-	-	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.
Hyaluronidase		+	-	+	-	-	N.D.	N.D.	N.D.	N.D.	N.D.	N.D.

(+), activity present; (-), activity absent; (N.D), not determined.

3.3. Purification of *Bm*K venom hyaluronidase BmHYA1

3.3.1 Gel filtration chromatography

A total of 10 fractions were separated by Superdex G75 gel filtration chromatography from BCV. The high molecular weight fractions were eluted mainly in the first 4 peaks, with the highest hyaluronidase activity found in peak 2 (Fig. 3.16). Peak 2 was collected (indicated by the horizontal bar) for further separation.

3.3.2 Anion exchange chromatography

Peak 2 was subsequently applied to anion exchange chromatography using UNOQ1 column, equilibrated with 50 mM NH_4HCO_3 solution, pH 8.9. The column was eluted with a linear gradient of 1 M NaCl. Peak 2 was resolved into six peaks, with peak 1 (unbound part) showing hyaluronidase activity (Fig. 3.17). The unbound component was collected (indicated by the horizontal bar) and lyophilized for further separation.

3.3.3 Cation exchange chromatography

The lyophilized components were re-dissolved in sodium acetate buffer (50 mM, pH 5.0) and subjected to cation exchange chromatography on UNOS1 column. A linear NaCl gradient elution resolved the components further into 3 peaks, with the peak 3 found to be positive for hyaluronidase activity (Fig. 3.18).

3.3.4 RP-HPLC

Finally, peak 3 (indicated by a horizontal bar in Fig 3.18) was applied on a Sephasil C_8

RP-HPLC column and a single peak with hyaluronidase activity was collected after elution with 0.1% TFA in 80% acetonitrile (Fig. 3.19). A summary of the purification process is shown in Table 3.2. From 200 mg of crude venom, 0.32 mg of purified hyaluronidase was obtained, with a yield of 12.4%. Specific activity increased by 77.5-fold after purification.

Table 3.2. Summary of purification process

Purification stage	Protein (mg)	Specific activity (units/mg)	Total activity (units)	Purification (fold)	Yield (%)
Crude venom	200	244	48,800	1.0	100
Superdex G75	21.6	1,366	29,505	5.6	60.5
UNOS1	0.91	9,010	8,199	36.9	16.8
RP-HPLC C_8	0.32	18,900	6,048	77.5	12.4

3.4. Homogeneity and molecular weight of BmHYA1

The homogeneity of BmHYA1 was indicated by a single band of SDS-PAGE, as shown in Fig.3.20. Based on the M.W. of protein standards, the M.W. of BmHYA1 was estimated to be 48 kDa. MALDI-TOF MS showed a symmetric peak (Fig. 3.21), indicating a M.W. of 48,696 Da. The results of SDS-PAGE and MALDI-TOF MS were found to be consistent. In addition, no other peptide contaminants other than the target protein were detected during the automated Edman degradation process.

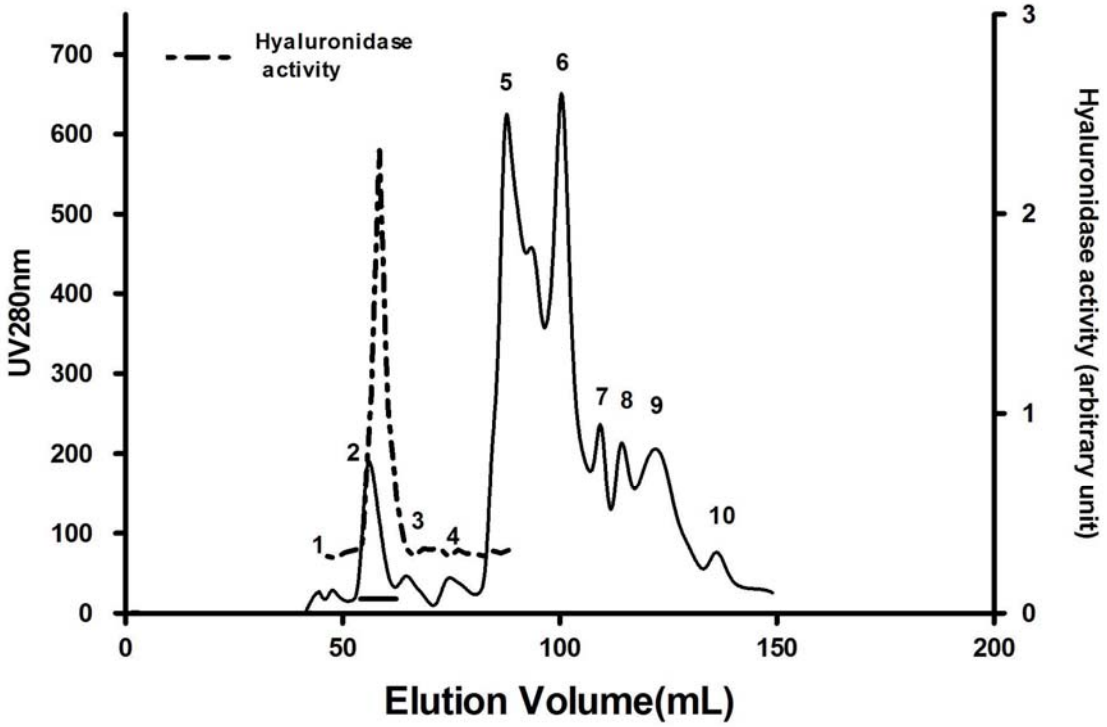

Fig. 3.16. Superdex G75 gel filtration chromatogram. The elution buffer was 50 mM NH_4HCO_3, pH 8.9. Out of the 10 peaks eluted from the column, peak 2 was associated with hyaluronidase activity and was collected for further separation (indicated by a horizontal bar).

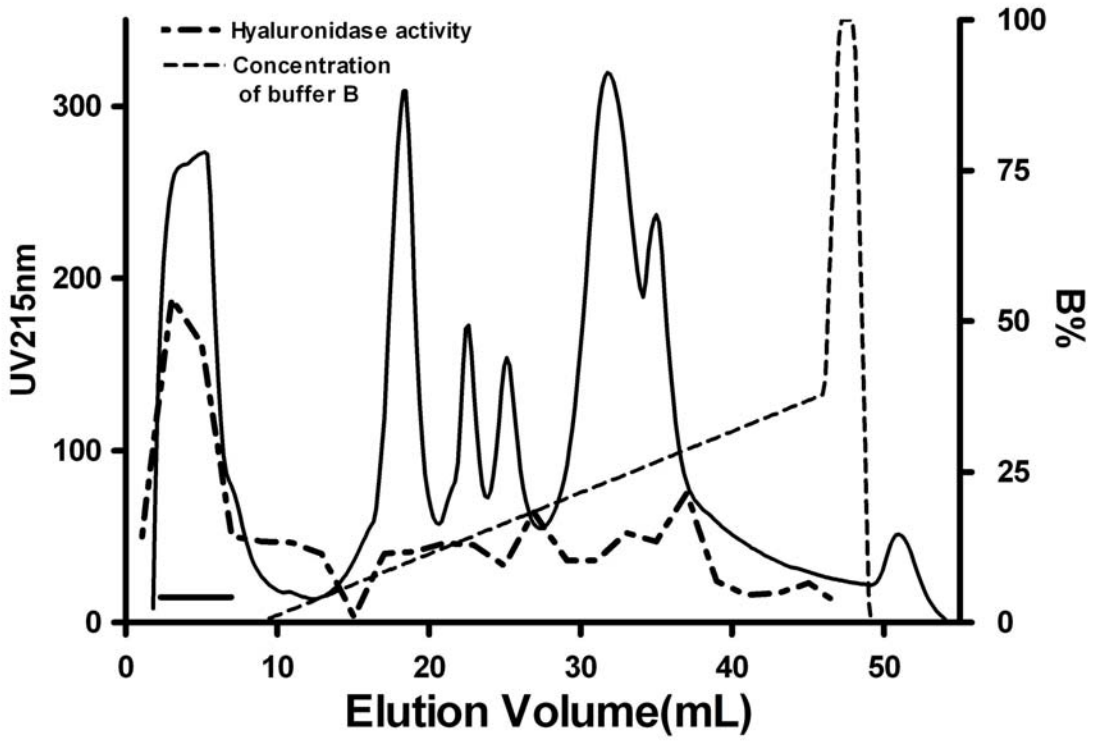

Fig.3.17. UNO Q1 anion exchange chromatogram. The column was pre-equilibrated with 50 mM NH_4HCO_3, pH 8.9 buffer. The pooled fractions associated with peak 2 (collected from above gel filtration) were loaded and eluted with a linear gradient of 1 M NaCl. The unbound fraction containing hyaluronidase activity (indicated by a horizontal bar) was pooled and further separated on cation exchange column.

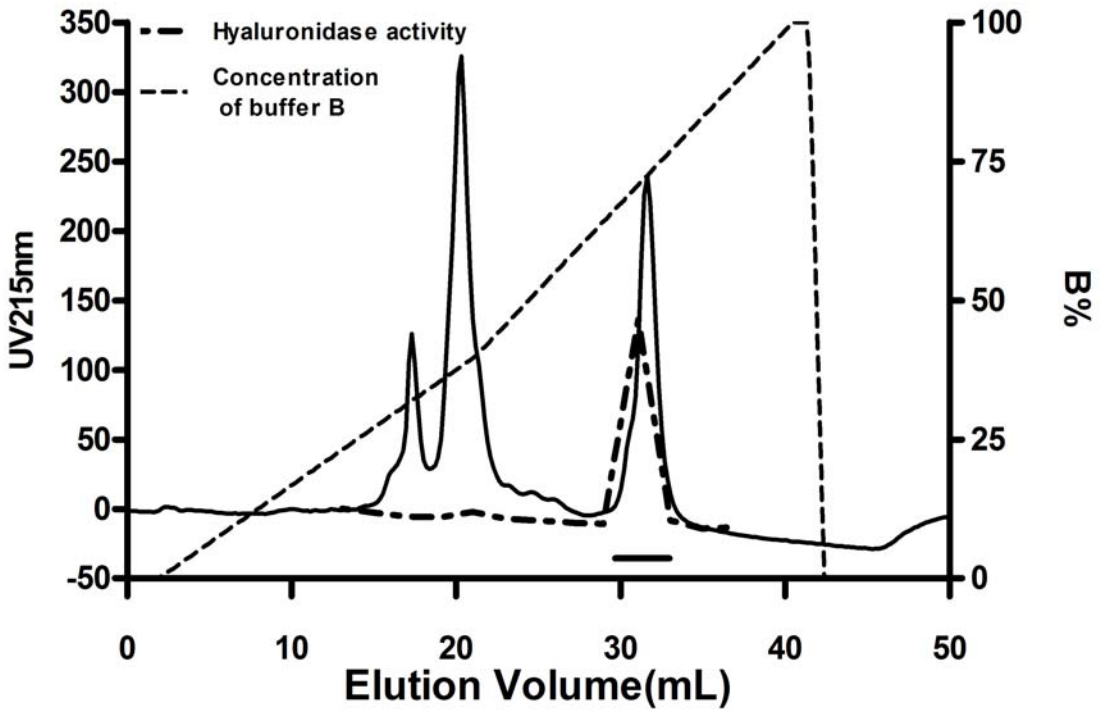

Fig.3.18. UNO S1 cation exchange chromatogram. The unbound fraction from UNO Q1 column was lyophilized and re-dissolved in sodium acetate (50 mM, pH 5.0), and fractionated on the cation exchange column UNOS1. The column was pre-equilibrated with 50 mM sodium acetate buffer, pH 5.0, and the elution was achieved with a linear gradient of 1 M NaCl. The last peak containing hyaluronidase activity (indicated by a horizontal bar) was collected for the further separation.

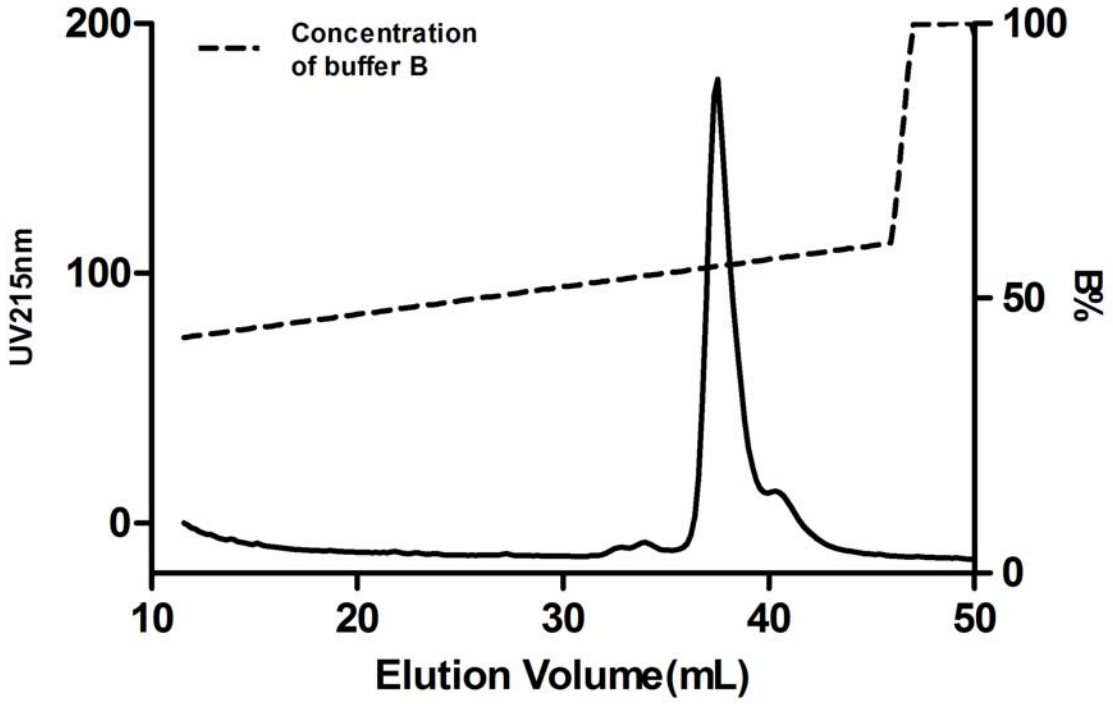

Fig.3.19. RP-HPLC chromatogram of BmHYA1. The pooled fraction obtained from UNO S1 cation exchange chromatographic step was purified on RP-HPLC Sephasil C_8 column equilibrated with buffer A (0.1% TFA). Elution was done by applying a linear gradient of buffer B (0.1% TFA in 80% acetonitrile). The protein was eluted at a very high acetonitrile concentration with the identification of hyaluronidase activity.

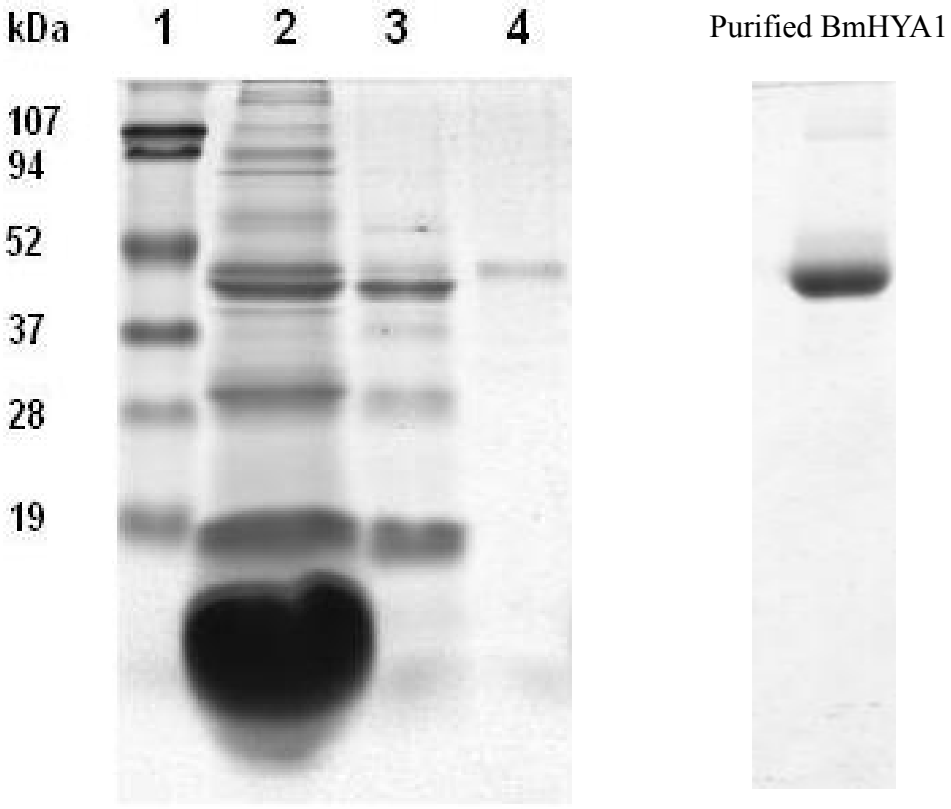

Fig. 3.20. SDS-PAGE of BmHYA1. Left Panel: Lane 1, protein standards; lane 2, crude venom of *Bm*K*;* lane 3, gel filtration peak 2; lane 4, BmHYA1. Left panel showed the apparent M.W. of BmHYA1 (lane 4) to be approximately 48 kDa. Right panel: purified BmHYA1.

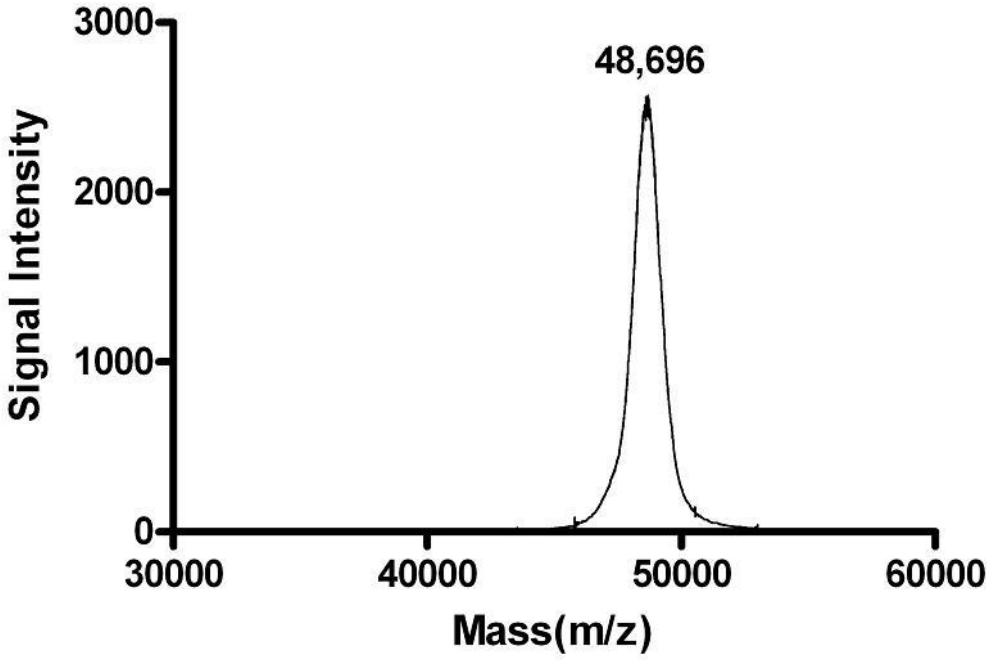

Fig.3.21. MALDI-TOF MS showed the M.W. of BmHYA1 to be 48696 Da, which was consistent with the M.W. estimated from SDS-PAGE profile. The single symmetric peak also confirmed the homogeneity of BmHYA1.

3.5 N-terminal sequence of BmHYA1

Thirty N-terminal amino acids of BmHYA1 were determined by Edman degradation to be: TSADF-KVVWE-VPSIM-CSKKF-KICVT-DLLTS. The N-terminal amino acid sequence of BmHYA1, and the hyaluronidases from honeybee and stonefish (SFHYA1) venom were then compared. The N-terminal sequence of BmHYA1 shared about 30% identity to honeybee venom hyaluronidase and 16% to SFHYA1 (Fig. 3.22 (A)). It is of interest to note that the two cysteine positions in the BmHYA1 N-terminal sequence were located exactly as the ones in the N-terminal sequence of the scorpion *Tityus stigmurus* hyaluronidase (Fig. 3.22 (B)).

3.6 Optimal pH profile

The acidic environment was essential for BmHYA1 enzyme activity. As demonstrated in Fig.3.23, the optimal pH of BmHYA1 was 4.5. The enzyme showed little activity at pH values below 2 or above 10.

3.7 Optimal temperature profile

The optimal temperature of BmHYA1 was 50 ^{o}C, as demonstrated in Fig. 3.24. Since most of the venom hyaluronidases show their optimal temperature around 37 ^{o}C (Kemparaju and Girish, 2006; Pessini et al., 2001; Girish et al., 2002; Morey et al., 2006), a venom and a mammalian tissue hyaluronidase with known optimal temperature were used for comparison with that of BmHYA1. It was noted that while the activity of the *D. russelii* venom hyaluronidase declined considerably at 50 ^{o}C, the optimal temperature for

Hyaluronidases	Thirty N-terminal amino acids	Ref.
BmHYA1	---------TSADFKVVWEVPSIMCSKKFKICVTDLLTS	Present study
Honeybee Hyaluronidase	------NNKTVREFNVYWNVPTFMC-HKYGLRFEEVS--	Gmachl and Kreil, 1993
SFHYA1	LPWTDPPLHPGHPFLFTWNAPTELC----GIRFG-----	Ng et al., 2005

Fig. 3.22. (A)
Comparison of thirty N-terminal amino acids of three venom hyaluronidases. The symbol letters in frame indicated the same positions of the three hyaluronidases. The table showed BmHYA1 shared up to approximately 30% of identity to other venom hyaluronidases based on the thirty-length N-terminal amino acid sequences of the scorpion venom hyaluronidases.

Source	5	10	15	20	25	30	Ref.
1.*Bm*K (scorpion)	TSADF	KVVWE	VPSIM	CSKKF	KICVT	DLLTS	Present study
2.*Tityus stigmurus* (scorpion)	EHPAL	YRRYS	KEHTF	CKTKN	QXCN		Batista et al., 2007

Fig. 3.22. (B)
Comparison of N-terminal sequences of BmHYA1 and *T.stigmurus* scorpion venom hyaluronidase. Two cysteine positions at the 16th and 23rd amino acids of BmHYA1 were found to be identical with those of the hyaluronidase from *Tityus stigmurus* scorpion venom. The lysines also appeared at the same position (the 19th amino acid).

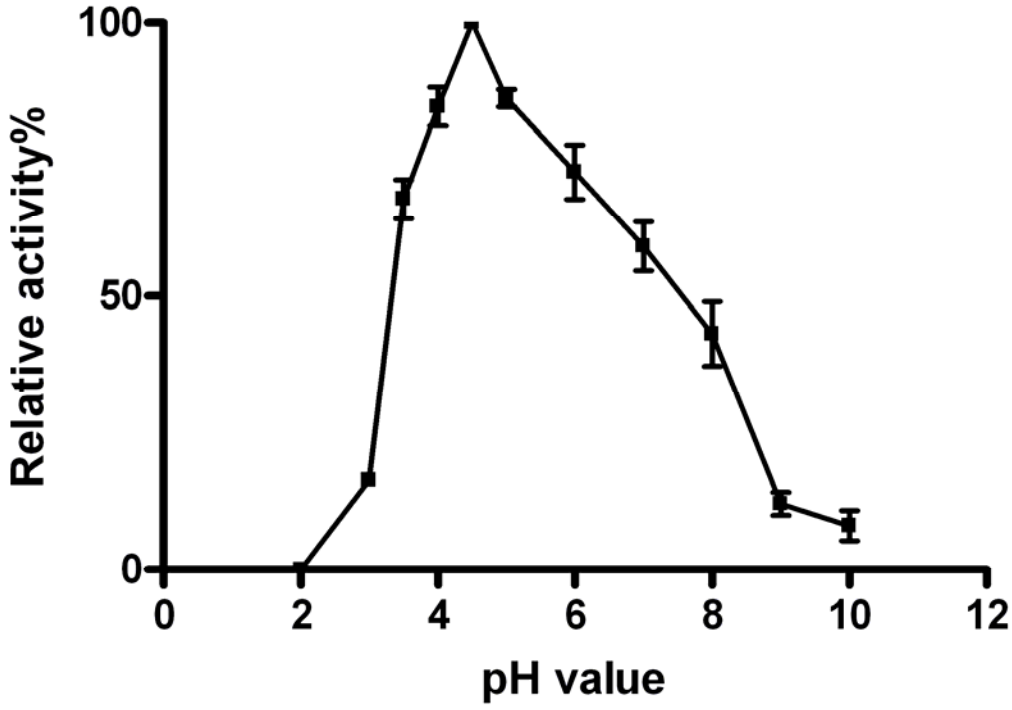

Fig. 3.23. Optimal pH profile of BmHYA1. Different buffer systems were used to provide varying pH settings. Maximal activity was set at 100% and the baseline activity at 0%. The reaction was conducted at 37 ^{o}C.

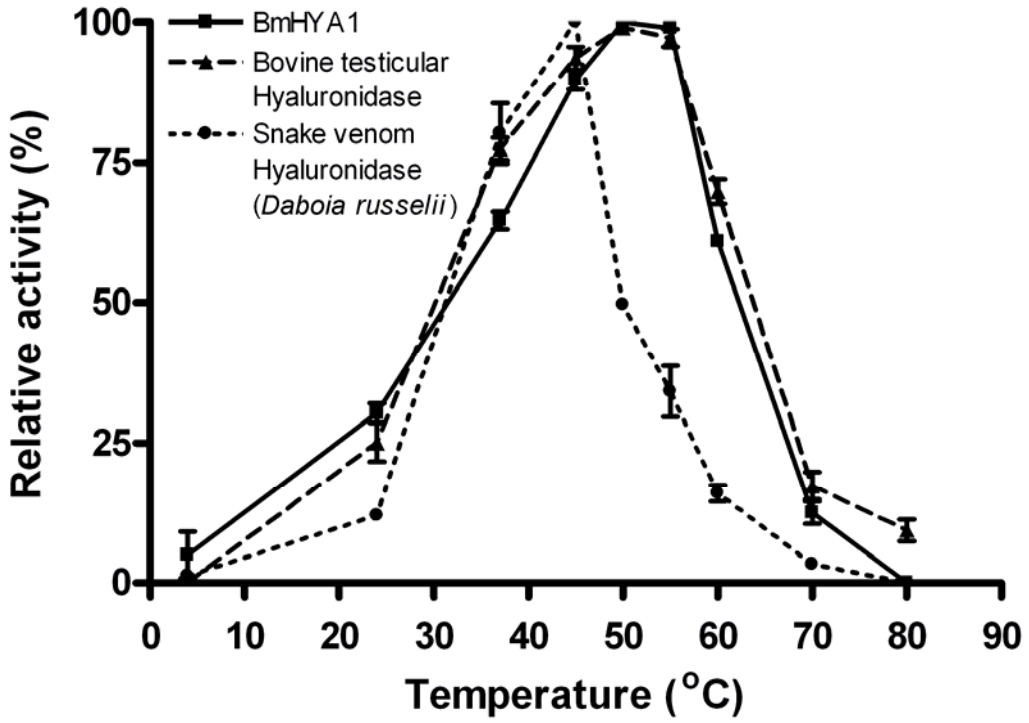

Fig.3.24. Optimal temperature profile of BmHYA1. The optimal temperature profile of BmHYA1 was compared with hyaluronidases from bovine testis and *D.russelii* venom. BmHYA1 and bovine testicular hyaluronidase shared similar patterns.

both BmHYA1 and bovine testicular hyaluronidase remained same at 50 ^{o}C.

3.8 Thermostability

BmHYA1 was pre-incubated in various temperatures for 10 minutes before its activity was tested. Its activity was found to be unstable to the temperature change. As shown in Fig. 3.25, the activity declined slightly before reaching 24 °C, but began losing its activity rapidly from 24 °C onwards. At 65 °C, the enzyme was totally inactivated.

3.9 K_m and V_{max} determinations

As demonstrated in Fig.3.26, K_m and V_{max} were determined as 95.3 μg/mL and 3.9 μg/min, respectively. See section 2.5.6 for further details about K_m and V_{max}.

3.10 Inhibition assays

Among the chemicals examined, heparin, DTT, Cu^{2+}, and Fe^{3+} showed inhibitory effect on BmHYA1, with the strongest effect found for heparin, which can reduce about 80% of the activity. Reduced glutathione, L-Cysteine, EDTA, Mg^{2+} and Ca^{2+} failed to demonstrate any detectable inhibitory effect on BmHYA1 enzyme activity (Table 3.3).

3.11 Deglycosylation assays

Assessing the degree of deglycosylation of BmHYA1 was by the mobility shifts on SDS-PAGE. As shown in Fig. 3.27, the extent of deglycosylation for BmHYA1 was observed by sequential addition of each of the three enzymes PNGase F, α-2(3,6,8.9)-Neuraminidase, and O-Glycosidase. The noticeable shift of BmHYA1 bands between

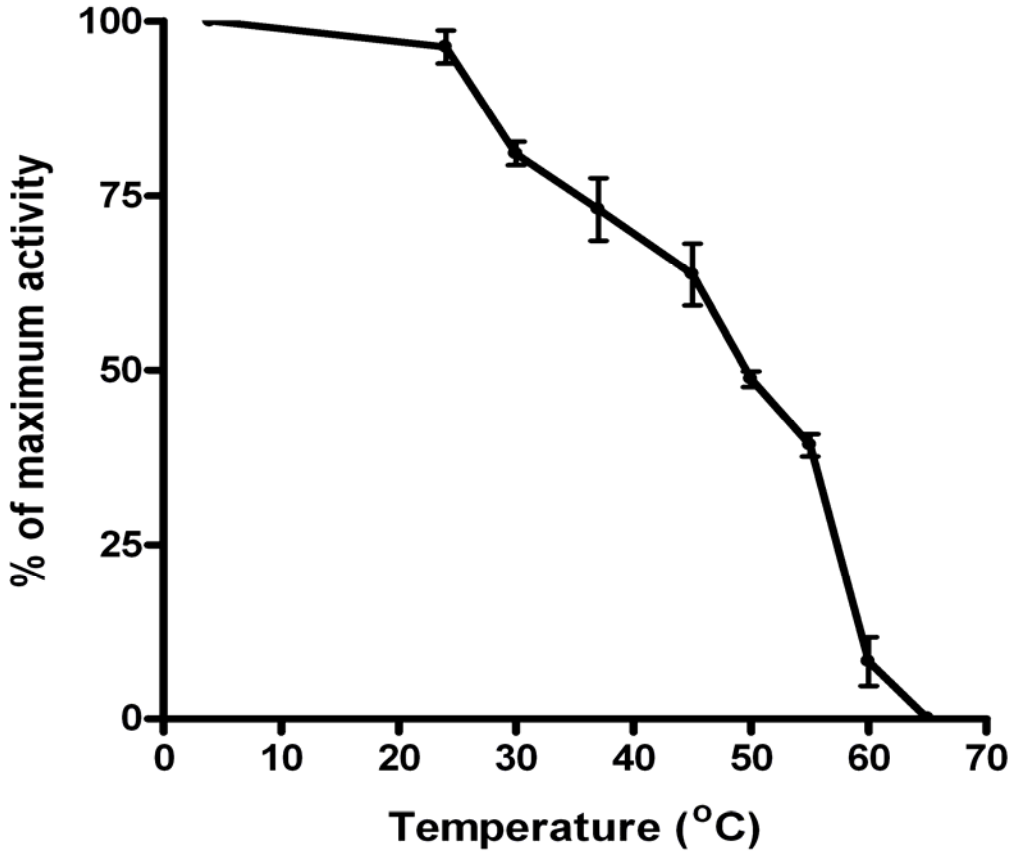

Fig.3.25. Effect of temperature on BmHYA1.

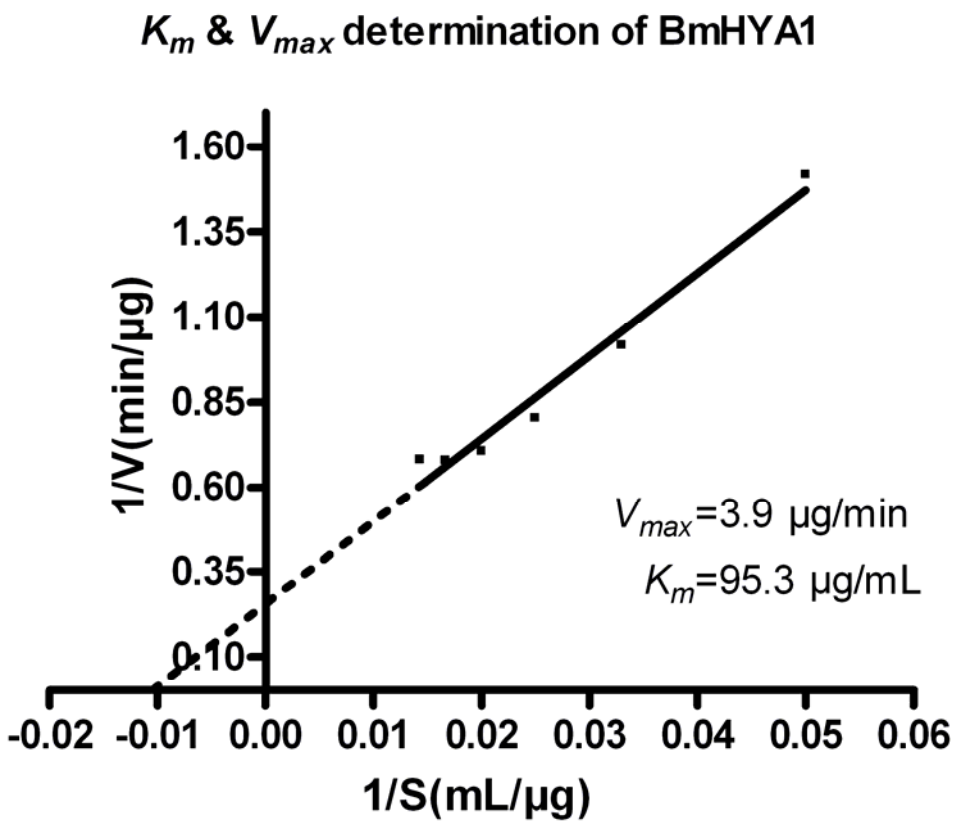

Fig.3.26. Lineweaver–Burk plot for determination of K_m and V_{max} values.

Table 3.3. The inhibitory effect of different chemicals on BmHYA1.*

Compounds	% Inhibition
Ions	
$MgCl_2$	N.D.
$CaCl_2$	N.D.
$CuCl_2$	9.9±1.43
$FeCl_3$	41.3±5.20
Others	
Glutathione (Reduced)	N.D.
L-Cysteine	N.D.
EDTA	N.D.
DTT	7±2.26
Heparin	81.6±6.05

* All chemicals were used at a fixed concentration of 5 mM, except heparin which was used at 1.5 mg/mL. N.D.: not detectable. The results are expressed as mean ± S.E.M..

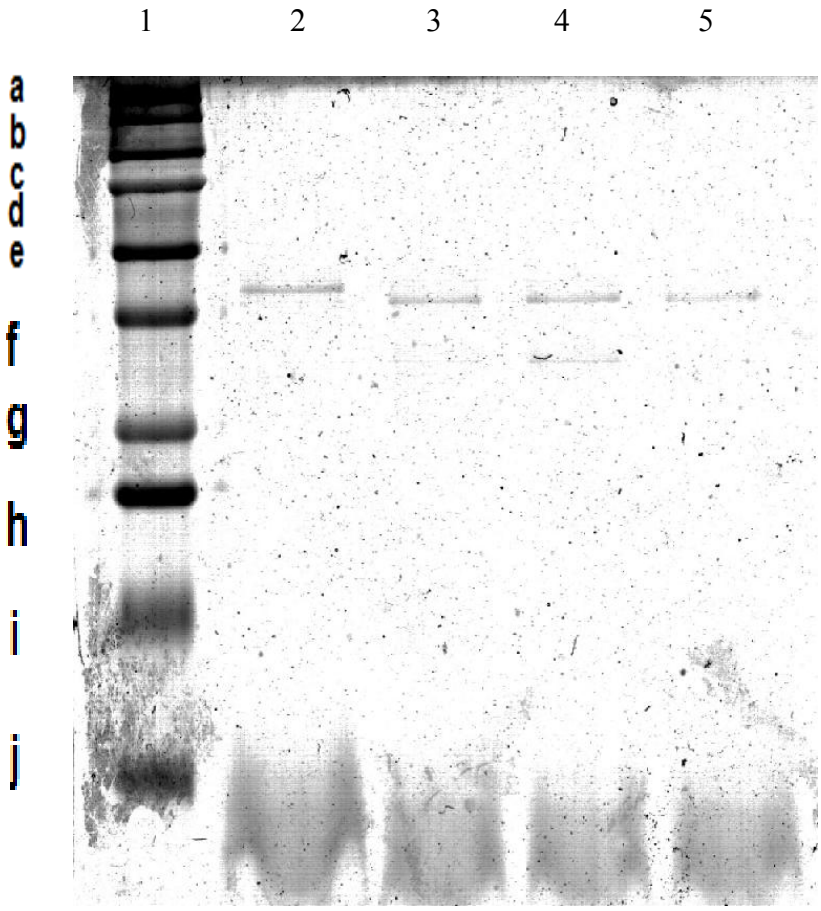

Fig. 3.27. Deglycosylation analysis under reducing conditions by 15% SDS-PAGE. Lane 1: protein standards (kDa): (a)250, (b)150, (c)100, (d)75, (e)50, (f)37, (g)25, (h)20, (i)15 and (j)10; lane 2: BmHYA1 only; lane 3: BmHYA1+ PNGase F; lane 4: BmHYA1 + PNGase F + O-Glycosidase; lane 5: BmHYA1+ PNGase F + O-Glycosidase + α-2(3,6,8,9) neuraminidase. A shift of two BmHYA1 bands between lane 2 and any of lane 3, 4 or 5, was evident after PNGase treatment. No obvious shift of BmHYA1 bands was observed among lanes 3, 4 and 5.

lane 2 and any of land 3, 4 or 5 was observed, suggesting the removal of *N*-linked oligosaccharides by PNGase F. Among lanes 3, 4 and 5, no obvious shift was seen, indicating that BmHYA1 contains no or very small amount of *O*-linked oligosaccharides.

3.12 End products of hydrolysis of hyaluronan by BmHYA1

The hyaluronidase enzymes hydrolyze the glycosidic bond of hyaluronan and subsequently generate oligosaccharides. In this study, the degradation product of hyaluronan by bovine testicular hyaluronidase, which is known as tetrasaccharide, was used as the marker of molecular size (Fig. 3.28 lane 1) to compare with that degraded by BmHYA1. Hyaluronan was incubated with BmHYA1 for 24 hours to obtain the completely digested substrate. Incubation of the hyaluronan and BmHYA1 for 24 hours showed the same pattern as the degradation product by bovine testicular hyaluronidase (Fig. 3.28 lane 2). The results suggested that tetrasaccharide was the main product when hyaluronan was completely degraded by BmHYA1.

3.13 The molecular biological study

3.13.1 RNA isolation and integrity test

Total RNA was isolated from the pair of venom glands of a single *Bm*K scorpion. Totally 28 μL of 82 ng/μL total RNA (approximately 2.3 μg) was yielded. The integrity was tested by native agarose gel (1.5%). The electrophoresis of total RNA in agarose gel showed that the 28S and 18S bands were clear, with 18S band appeared to be brighter and broader than the 28S band. Neither the smaller band nor any smeared area was detected

Fig.3.28. Thin layer chromatogram of degradation products of hyaluronan by BmHYA1. Hyaluronan was incubated with Bovine testicular hyaluronidase and with BmHYA1 for 24 hours separately. From the chromatogram, the degradation products of hyaluronan by BmHYA1 (lane 2) showed a similar chromatographic pattern to the degradation products of hyaluronan by bovine testicular hyaluronidase (lane 1).

below the 18S band. The integrity of the RNA sample was thus confirmed for further processing.

3.13.2 RT-PCR and 3' RACE

Using AP, ss cDNA was synthesized from total RNA. RNA template was degraded by RNase H. The 3' RACE PCR was primed with BmHYA1-GSP and AUAP. The 3' RACE product of rat GAPDH primer and AUAP was found to contain a smeared area spanning 500 to 2000 bp region (Fig. 3.29, lane 2), thus non-specific binding might have probably occurred. It appeared likely that the nucleotide sequences between rat and *Bm*K scorpion GAPDHs was different. The 3' end of *Bm*K AS-1 from the primer was about 300 bp, and based on the length of this cDNA, the band of approximately 300 bp detected in lane 3 indicated that it was most probably the 3' end of *Bm*K AS-1. It also confirmed the good quality of this cDNA material. Lane 4 also showed a smeared area, which might be due to the unspecific binding of SFHYA1-GSP to BmHYA1 sequence. A band of approximately 1.3 kb and a smeared area underneath were detected in lane 5. A degenerate primer containing a mixture of various primers commonly generates a heterogeneous product. Judging by the molecular size (48 kDa) of BmHYA1, this 1.3 kb band might probably represent the BmHYA1 3' end of cDNA. Hence, it was excised and purified from the gel for further study.

3.13.3 TA cloning of the 1.3 kb fragment

The fragment was subcloned into Promega pGEM T Easy vector, and the ligated

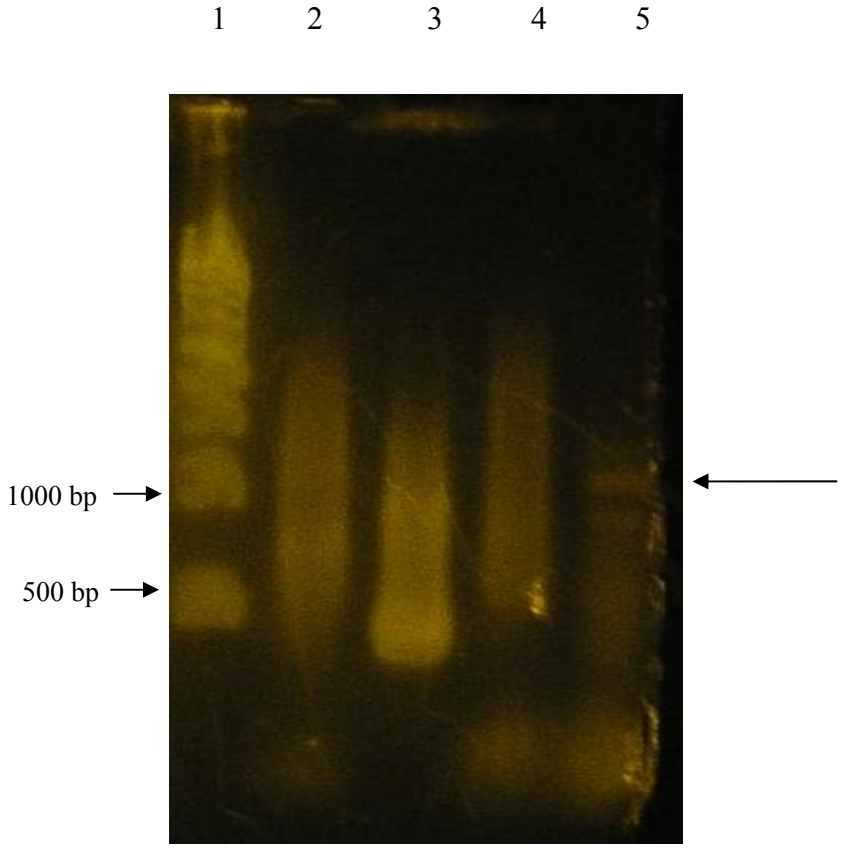

Fig. 3.29. Electrophoresis for 3' RACE PCR product. Lane1: 500 bp ladder; lane2: rat GAPDH primer and AUAP PCR product; lane3: *Bm*K AS-1 primer and AUAP PCR product; lane 4: hyaluronidase universal primer and AUAP PCR product; lane 5, BmHYA1-GSP and AUAP PCR product. All of the four PCR reactions were conducted at the same time. Arrow: expected BmHYA1 fragment (approximately 1.3 kb).

product was transformed into *E.coli* strain DH5α competent cells. After incubation at 37 ^{o}C overnight, 20 positive (white) clones were picked. Out of the selected clones, 10 were found to be successfully inserted with the target fragments (for methods see section 2.6.4.5) and were applied for sequencing. The representative result for the first 5 selected clones was shown in Fig 3.30. Clones F2 and F3 were tested to contain the sequences belonging to Glycol-Hydro-56 family.

3.13.4 3' end cDNA nucleotide and full length protein sequences of BmHYA1

Nucleotide sequence and deduced amino acid sequence of BmHAY1 were listed in Fig.3.31. The 3' end of cDNA of BmHYA1 gene was cloned. The translational termination codon of this cDNA was assigned to the TGA codon (marked as asterisk in Fig. 3.31). The downstream of this TGA codon, a putative polyadenylation signal of AATAAA was observed (underlined by a wavy line in Fig. 3.31). The DNA sequence containing stop codon and poly A tail indicates the completion of 3' UTR. Since BmHYA1-GSP was designed based on the known N-terminal protein sequence, the deduced protein sequence along with the Edman degradation result provided the whole amino acid sequence of mature BmHAY1 protein. BmHYA1 mature protein contains 385 amino acids including 12 cysteines. Among 10 successful clones, the sequences have some discrepancies: clone F15, T for A at position 77; clone F2, G for W at position 107, F for L at position 110; clone F10, G for W at position 210. The predominant sequence was picked as the standard. Between experimental and theoretical sequences, only one mismatched position was found. Cysteine at position 23rd of N-terminal amino acid sequence was found out to be asparagine as deduced from the nucleotide codon.

Since BmHYA1 is the first scorpion venom hyaluronidase ever characterized at the molecular level, it is significant to investigate its structure relationship to other known hyaluronidases.

BmHYA1 was aligned with other hyaluronidases using Cluster W algorithm (Thompson et al., 1994, Fig.3.32). In addition, a phylogenetic tree was generated to further investigate the relationship of BmHYA1 to its 26 homologues (all belong to glycol-hydro-56 superfamily), including the hyaluronidases of *C.elegans* and representative of mammals, snakes, insects and fish (Fig. 3.33). Among these homologues, the sequence identity of BmHYA1 to any of the other hyaluronidases did not exceed 34%. The closest BmHYA1 homologues are hyaluronidases of white-face hornet and yellow jacket wasp (both 34% identity) In the tree, *C. elegans* formed a cluster separated from all other hyaluronidases. Similarly, BmHYA1 was also located distantly to other hyaluronidases and formed a separate cluster. The phylogenetic analysis indicates early divergence and independent evolution of BmHYA1 from other members.

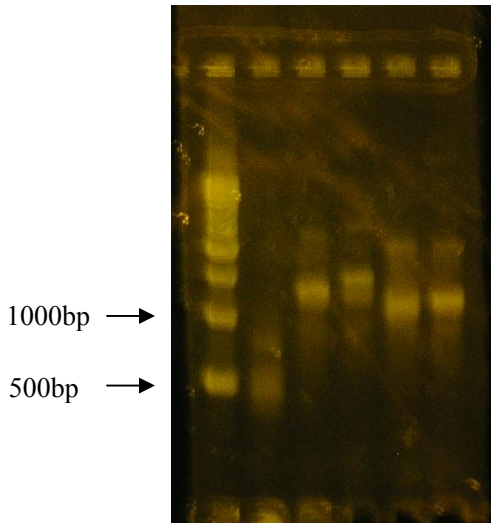

Fig. 3.30. The verification of inserts. The PCR products (using T7 and SP6 as primers) were examined by agarose gel electrophoresis. From left to right: DNA ladder (500 bp), F1, F2, F3, F4, F5. F2~F5 showed the expected sizes (around 1.3 kb) and then were sent for sequencing. As a result, F2 and F3 were tested to contain the sequences belonging to Glycol-Hydro-56 family.

```
                                                T  S  A          3

GACTTTAAGGTGGTTTGGGAGGTTCCTTCGATAATGTGTAGCAAAAAGTTT          51
D  F  K  V  V  W  E  V  P  S  I  M  C  S  K  K  F            20

AAAATTAATGTTACAGATTTGCTTACAAGCCACAAAATATTGGTCAATCAA          102
K  I  N  V  T  D  L  L  T  S  H  K  I  L  V  N  Q            37

GAAGAGACGTTTAACGGAGATAAAATTGTAATCTTCTATGAGAGCCAACTC          153
E  E  T  F  N  G  D  K  I  V  I  F  Y  E  S  Q  L            54

GGAAAATATCCTCATATAGAATCTCATGGAGATATCAATGGAGGGATGTTG          204
G  K  Y  P  H  I  E  S  H  G  D  I  N  G  G  M  L            71

CAAGTTTCAGATTTAGCGAATCATTTAAAGATTGCGAGAGATAACATATCG          255
Q  V  S  D  L  A  N  H  L  K  I  A  R  D  N  I  S            88

AAATTTATACCCGATCCAAATTTTAATGGAGTTGGAATTATAGACTGGGAA          306
K  F  I  P  D  P  N  F  N  G  V  G  I  I  D  W  E            105

GCATGGCGTCCTCTCTGGAAATACAATTGGGGTAGGATGAGTGAGTACAGG          357
A  W  R  P  L  W  K  Y  N  W  G  R  M  S  E  Y  R            122

GATAGATCAAAAGATCTTGTAAAAGCTAAACATCCAGATTGGTCACCGGCT          408
D  R  S  K  D  L  V  K  A  K  H  P  D  W  S  P  A            139

CAAATCGAAAAAGTTGCTATAGAGGAATGGGAAAACAGTGCCAAAGAGTGG          459
Q  I  E  K  V  A  I  E  E  W  E  N  S  A  K  E  W            156

ATGCTTAAAACACTGAAGCTTGCAGAAGATATGAGGCCTAATGCAGCATGG          510
M  L  K  T  L  K  L  A  E  D  M  R  P  N  A  A  W            173

TGTTACTATCTGTTTCCCGACTGTTATAACTATGGTGGGAAAGATCAACCT          561
C  Y  Y  L  F  P  D  C  Y  N  Y  G  G  K  D  Q  P            190

TCAGAATATTTTTGCAAGAATGATATACAAGAAGCAAACGACAAATTATCC          612
S  E  Y  F  C  K  N  D  I  Q  E  A  N  D  K  L  S            207

TGGTTGTGGAAACAAAGTACAGCTCTTTGCCCTTCGATATACATGCAAGAA          663
W  L  W  K  Q  S  T  A  L  C  P  S  I  Y  M  Q  E            224

TCGCACATAACGAAATATAACACATCTCAACGCGCATGGTGGATATATGCT          714
S  H  I  T  K  Y  N  T  S  Q  R  A  W  W  I  Y  A            241

CGTCTAAGGGAAACCATAAGATTATCACATCCTAATACTCTCATTTATCCA          765
R  L  R  E  T  I  R  L  S  H  P  N  T  L  I  Y  P            258
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TATATAAACTACATTTTGCCAGGAACTAAGAAGACGGTACCATCGATGGAT        816
Y  I  N  Y  I  L  P  G  T  K  K  T  V  P  S  M  D        275

TTTAAAAGAGTATTAGGACAGATAGGATCTCTGGGTCTGGACGGTGCTATC        867
F  K  R  V  L  G  Q  I  G  S  L  G  L  D  G  A  I        292

ATTTGGGGATCTTCTTATCATGTGAATACCGAAGGAATGTGTAAAGAAATG        918
I  W  G  S  S  Y  H  V  N  T  E  G  M  C  K  E  M        309

AAAACCTACGTGAAAGATGTGATTGCTCCTGTAGCTAGTACTGTGATACAA        969
K  T  Y  V  K  D  V  I  A  P  V  A  S  T  V  I  Q        326

AATGTCAATCGATGTAGTCAGCAAATCTGCAAAGGAAGAGGAAATTGTGTA       1020
N  V  N  R  C  S  Q  Q  I  C  K  G  R  G  N  C  V        343

TGGCCAGAAGAACCTTATACCTCATGGAAGTATCTGATCGATCCCAAAAAT       1071
W  P  E  E  P  Y  T  S  W  K  Y  L  I  D  P  K  N        360

CCGACATTCAAACATACTAACATCTCTTGCAAATGCAAAGGTGGTTATACG       1122
P  T  F  K  H  T  N  I  S  C  K  C  K  G  G  Y  T        377

GGACGTTATTGTCAAATTGCACCTTGAGATTTTCTACCTGATCGCAATACT       1173
G  R  Y  C  Q  I  A  P  *                                 385

GTATAAGATTAATATATTCGATAACGAATTATTATTTAAATGCAAATTTTT       1225
AAAATGTTTTTAATAATTTAATTTACGAGAATTATGTAAAATAAAGTGGTT       1277
AATATTAAAAAAAAAAAAAAAA                                    1300
```

Fig. 3.31. Full length deduced amino acid sequence of BmHYA1 and its molecular characterization. Amino acid residues including the first 3 N-terminal amino acid residues (T-S-A) obtained by Edman degradation are underlined. The boldfaced amino acid residues indicate the sequence used for designing the degenerate primer. The five potential glycosylated asparagines are shaded. The stop codon TGA is indicated by an asterisk. The putative polyadenylation signal AATAAA is underlined by a wavy line.

```
                                                                                *      #
BmHYA1          ------------------------------------------------TS---ADFKVVWEVPSIMCSKKFKINVTDLLTS         30
Honeybee HYA    --------MSRPLVITEGMMIG----VLLMLAPINALLLGFVQSTPDNNKTVRE.N.Y.N..TF..H-.YGLRFEEVSEK         67
SFHYA1          --MIK---LKFLYVQHFFSIS-----VTMMSLLRSGGALPWTDPPLHPG---HP.LFT.NA.TEL.GIR.GMPLD--.SY         65
Snake Eo HYA    --------MYHLWIKCLA--------AWIFLKRFNGVHVMHAKAPMYPN---EP.L.F.NA.TTQ.RLRY.VDLD--.KT         59
HYAL1           --------MAGHLLP-----------ICALFLTLLDMAQGFRGPLLPN----RP.TT..NANTQW.LERHGVD.D--VSV         55
HYAL2           --------MRAGPGPTVT--------LALVLAVSWAMELKPTAPPIF.G---RP.V.A.D..TQD.GPRL.VPLD--.NA         59
HYAL3           --------MTTQLGP------------ALVLGVALCLGCGQPLPQVPE----RP.S.L.N...AH.EAR.GVHLP--.NA         54
HYAL4           MKVLSEGQLKLCVVQPVHLTS----WLLIFFILKSISCLKPARLPIYQR---KP.IAA.NA.TDQ.LI.YNLRLN--.KM         71
Human PH-20     MGVLK---FKHIFFRSFVKSSGVSQIVFTFLLIPCCLTLNFRAPPVIPN---VA.LWA.NA..EF.LG..DEPLD--MSL         72

                                                                        #                ♦ ●
BmHYA1          HKILVNQEETFNGDKIVIFYESQLGKYPHIES-----HGDINGGMLQVSDLANHLKIARDNISKFIPDPNFNGVGIIDWE        105
Honeybee HYA    YG..Q.WMDK.R.EE.A.L.DP--.MF.ALLKDPNGNVVAR...VP.LGN.TK..QVF..HLINQ...KS.P...V..F.        145
SFHYA1          FDFVSSTLKSATNQS.S...TDRF.VF.YVNEK---TGKMY...LP.LI..EQ.HEL.E.D.EYY..FNQLG-LAVL.F.        141
Snake Eo HYA    FH.VA.AND.LS.SAVT...PTH..I....DD----RGHFFH.IIP.NES.TK..DKSKSD.NRI..LKT.H.L.V....        135
HYAL1           FDVVA.PGQ..R.PDMT...S....T..YYTP----TGEPVF..LP.NAS.IA..ARTFQD.LAA..A.D.S.LAV....        131
HYAL2           FDVQASPN.G.VNQN.T...RDR..L..RFD.----AGRSVH..VP.NVS.WA.R.MLQKRVEHY.RTQESA.LAV....        135
HYAL3           LG.IA.RGQH.H.QNMT...KN...L..YFGP----RGTAH...IP.ALP.DR..AL.AYQ.HHSLR-.G.A.PAVL...        129
HYAL4           FPVIGSPLAKAR.QNVT...VNR..Y..WYT.----QGVP....LP.NIS.QV..EK.DQD.NYY..AED.S.LAV....        147
Human PH-20     FSFIGSPRINAT.QGVT...VDR..Y..Y.D.I---TGVTV...IP.KIS.QD..DK.KKD.TFYM.VD.LG-MAV....        148

                                                                              ◊ ♦     *
BmHYA1          AWRPLWKYNWGRMSEYRDRSKDLVKAKHPDWSPAQIEKVAIEEWENSAKEWMLKTLKLAEDMRPNAAWCYYLFPDCYN--        183
Honeybee HYA    S...IFRQ..ASLQP.KKL.VEV.RRE..F.DDQRV.QE.KRRF.KYGQLF.EE...A.KR...A.N.G..AY.Y...--        223
SFHYA1          E...Q.IR...SKDI..QY.IET.LK.NSSL.KEEAAGQ.KMAF.RA..KYF.RSIRIGKRL...RL.G...Y.E...YE        221
Snake Eo HYA    N...Q.DR...NKNV..N..IQFARDL..EL.ENK.RRL.KA.Y.KA..SF.RD..L...E...DGY.G...Y...Q.YD        215
HYAL1           ....R.AF..DTKDI..Q..RA..Q.Q....PAP.V.A..QDQFQGA.RA..AG..Q.GRAL..RGL.GF.G......--        209
HYAL2           D...V.VR..QDKDV..RL.RQ..ASR....P.DR.V.Q.QY.F.FA.QQF..E..RYVKAV..RHL.GF........HD        215
HYAL3           E.C...AG....RRA.QAA.WAWAQQVF..LD.QEQLYK.YTGF.QA.RAL.ED..RV.QAL..HGL.GF.HY.A.G.-G        208
HYAL4           Y...Q.AR..NSKDV..QK.RK.ISDMGKNV.ATD..YL.KVTF.E...AF.KE.I..GIKS..KGL.G...Y...H.YN        227
Human PH-20     E...T.AR..KPKDV.KN..IE..QQQNVQL.LTEATEK.KQ.F.KAG.DFLVE.I..GKLL...HL.G.........HH        228
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                        *                    ◊    ♦         #
BmHYA1          YGGKDQPSEYFCKNDIQEANDKLSWLWKQSTALCPSIYMQESHITKYNTSQRAWWIYARLRETIRLSHPNTL-----IYP      258
Honeybee HYA    --LTPNQPSAQ.EATTMQE...M...FESEDV.L..V.LRWNLT----SGE.VGLVGG.VK.AL.IARQM.TSRK-KVL.      296
SFHYA1          .KKDMAGYTGE.PAIEKDR.NE.L...RE....F....LELLLR---D.Q.ARQYVRH.IQ.S..V.KLPNSAYSIP.HA      298
Snake Eo HYA    .KT.GDQYTGK.PDIEMSR..Q.L...RE....F.NV.LEIILR---SSDNALKFVHH..K.SM.IASMAREDYALPVFV      292
HYAL1           .DFLSPNYTGQ.PSG.RAQ..Q.G...G..R..Y.....PAVLE---G.GKSQMYVQH.VA.AF.VAVAAGD-PNLPVL.      285
HYAL2           .VQNWESYTGR.PDVEVAR..Q.A...AE....F..V.LD.TLA---SSRHGRNFVSF.VQ.AL.VARTHHANHALPV.V      292
HYAL3           WHSMASNYTGR.HAATLAR.TQ.H...AA.S..F....LPPRLP---PAHH-QAFVRH..E.AF.VALVGHR-HPLPVLA      283
HYAL4           VYA--PNYSGS.PE.EVLR.NE.....NS.A..Y...GVWK.LG---DSENILRFSKF.VH.SM.I.TMTSHDYALPVFV      302
Human PH-20     .KK--PGYNGS.F.VEIKR..D.....NE....Y....LNTQQ----SPVAATLYVRN.V..A..V.KIPDAKSPLPVFA      302

                                            ♦           *                !        ▼    ▼
BmHYA1          YINYILPGTKKTVPS-MDFKRVLGQIGSLGLDGAIIWGSSYHVNTEGMCKEMKTYVKDVIAPVASTVIQNVNRCSQQICK      337
Honeybee HYA    .YW.KYQDRRD.DL.RA.LEAT.RK.TD..A..F......DDI..KAK.LQFRE.LNNELG.AVKRIAL.N.ANDRLTVD      376
SFHYA1          .VRPVYKDSTDNYM.EF.LVNTI.EAAA..AASVVC..DMSV.A..DS.FDARRHLEK.MN.YIMN.STATQL..KAL.Q      378
Snake Eo HYA    .ARPFY-AYTFEPLTQE.LVTTV.ETAAM.AA.IVF...MQYAS.VDS.QKV.K.MNGPLGRYIIN.TTAAKI..HAL.R      371
HYAL1           .VQIFY-D.TNHFLP-------------------------L-----ES.QAI.E.MDTTLG.FILN.TSGALL...AL.S      334
HYAL2           FTRPTY-SRRL.GL.E..LISTI.ESAA..AA.V.L..DAGYTTSTET.QYL.D.LTRLLV.YVVN.SWATQY..RAQ.H      371
HYAL3           .VRLTH-RRSGRFL.QD.LVQSI.VSAA..AA.VVL..DLSLSSS.EE.WHLHD.LV.TLG.YVIN.TRAAMA..H.R.H      362
HYAL4           .TRLGYRDEPLFFL.KQ.LVSTI.ESAA..AA.IV...DMNLTASKAN.TKV.QF.SSDLGSYIAN.TRAAEV..LHL.R      382
Human PH-20     .TRIVFTDQVLKFL.QDELVYTF.ETVA..AS.IV...TLSIMRSMKS.LLLDN.METILN.YIIN.TLAAKM...VL.Q      382

                    ▼                                           #      #  ▼ ▼                ▼
BmHYA1          GRGNCVWPEEPYTSWKYLIDPK-----------------------NPTFKHTNISCKCKGGYTGRY--------CQIAP      385
Honeybee HYA    VSVDQ.                                                                              382
SFHYA1          DQ.R..RKHW-DDDVFLHL..RRYRIEQQRGGG--PLTVTGDLSQDDVNWFDR.FD.M.YSEKPC.S--------ALTFN      447
Snake Eo HYA    KN.R..RKHS-DSNAFLHLF.ESFRIMVHANATEKKVIVKGKLELE.LIYLRE.FM.Q.YQ.WQ.L.--------.EEYS      442
HYAL1           .H.R..RRTS-HPKALL.LN.ASFS--IQLTPGGGPLSLRGALSLEDQAQMAVEFK.R.YP.WQAPW--------.-E--      400
HYAL2           .H.R..RRNP-SA.TFLHLSTNSFRLVPGHAPGEPQLRPVGELSWADIDHLQ.HFR.Q.YL.WS.EQ--------.-QWD      441
HYAL3           .H.R.ARRDPGQMEAFLHLW.D---------------GSLGDW--------KS-F..H.YW.WA.PT--------.-Q--      407
HYAL4           NN.R.IRKMW-NAPSYLHLN.ASYHIEASEDG---EFTVKGKASDTDLAVMADTF..H.YQ..E.AD--------.REIK      450
Human PH-20     EQ.V.IRKNW-NS.DYLHLN.DNFAIQLEKGG---KFTVRGKPTLEDLEQFSEKFY.S.YSTLSCKEKADVKDTDAVDVC      458
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BmHYA1                                                                    385
Honeybee HYA                                                              382
SFHYA1          VINKAVIN---KAPPACDG---------TARSDVHCDVNINF                477
Snake Eo HYA    IKDIRKI                                                   449
HYAL1           ---R---------KSMW                                         405
HYAL2           HRQAAGGA-----SEAWAG---------SHLTSLLALAALAFTWTL            473
HYAL3           --EPRPGP-----KEAV                                         417
HYAL4           TADGCSGVS--PSPGSLMT---------LCLLLLASYRSIQL                481
Human PH-20     IADGVCIDAFLKPPMETEEPQIFYNASPSTLSATMFIVSIWFLIISSVASL       509
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Fig. 3.32. Comparison of hyaluronidase sequences by Clustal W algorithm (Thompson et al., 1994). The sequences include: BmHYA1 (present work), Honeybee HYA (honeybee venom hyaluronidase, AAA27730.1), SFHYA1 (stonefish venom hyaluronidase, AAO74499.1), Snake Eo HYA (snake *E. ocellatus* venom hyaluronidase, ABI33937.1), HYAL1~4 (human hyaluronidases 1~4, CAG46731.1, NP_149348.2, NP_003540.2, NP_036401.2) and Human PH-20 (AAC60607.2). The proposed catalytic Glu H-donating residue is denoted by "●"; the residues positioning the carbonyl nucleophile/base are denoted by "♦". The conserved cysteines which have the counterparts in Honeybee HYA, i.e., Cys^{16}, Cys^{181}, Cys^{195} and Cys^{306}, are identified by "*"; other conserved cysteines (Cys^{331}, Cys^{336}, Cys^{342}, Cys^{370}, Cys^{372} and Cys^{381}) are noted by "▼". Cysteines which may not be conserved in BmHYA1 sequence (Cys^{174} and Cys^{217}) are indicated by "◊". Potential *N*-glycosylation sites (N^{23}-V-T, N^{86}-I-S, N^{231}-T-S, N^{360}-P-T, N^{367}-I-S) are marked as "#" above the corresponding asparagine residues. The highly conserved potential hyaluronidase *N*-glycosylation sites are marked by "!" at position Thr^{323} of BmHYA1 sequence to indicate the first residue of the site. Residues hidden as "." are identical to the BmHYA1 sequence.

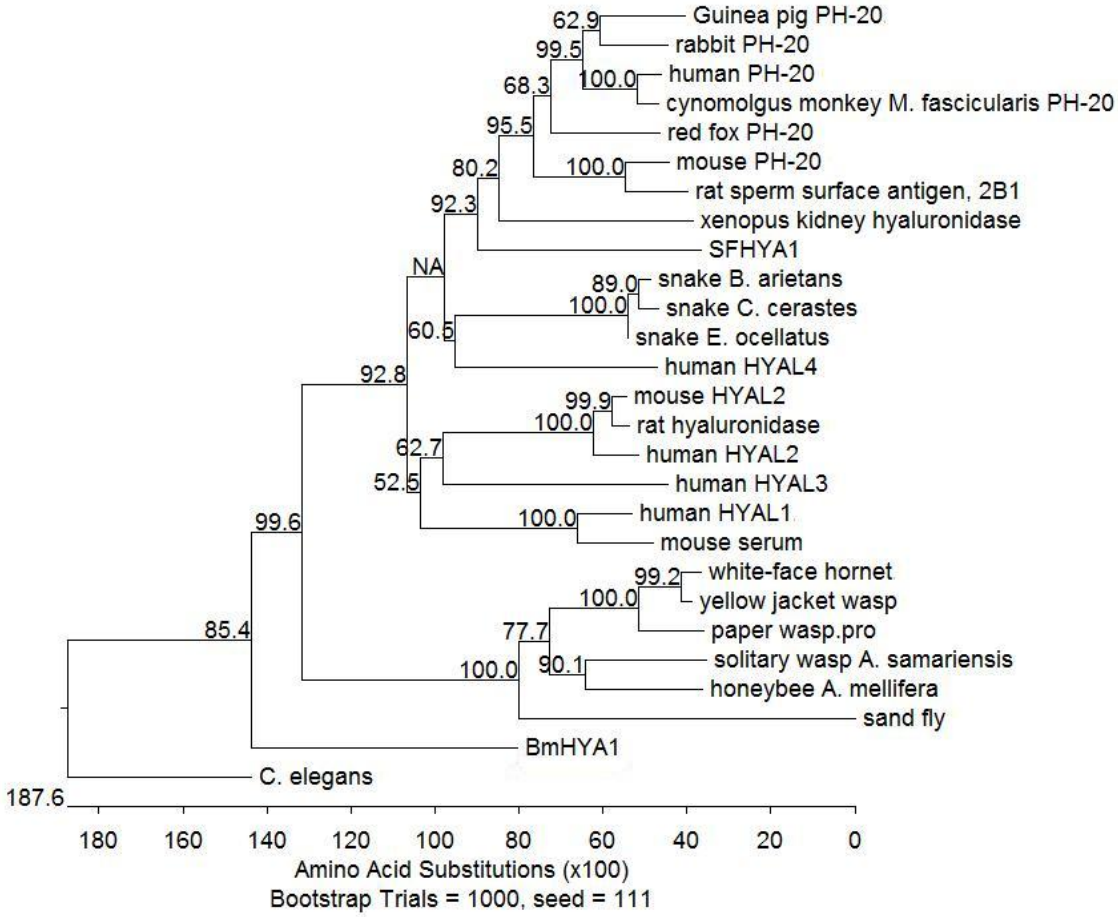

Fig. 3.33. Phylogenetic tree of BmHYA1 and other 26 hyaluronidases. The protein sequences were aligned by Clustal W algorithm (Thompson et al., 1994) and the phylogenetic tree was constructed by MegAlign (Lasergene, DNASTAR, Madison, WI). The sequences involved in phylogenetic analysis are: BmHYA1 (this work), *C. elegans* hyaluronidase (CAA88874.1), sand fly hyaluronidase (AAD32195.1), honeybee (*Apis mellifera*) venom hyaluronidase (AAA27730.1), paper wasp hyaluronidase (AAD52616.1), white-face hornet hyaluronidase (AAA68279.1), yellow jacket wasp hyaluronidase (AAB48073.1), solitary wasp *A. samariensis* (BAF93867.1), mouse serum hyaluronidase (AAC15949.1), human HYAL-1~4 (CAG46731.1, NP_149348.2, NP_003540.2, NP_036401.2), snake *C. cerastes* venom hyaluronidase (ABI33940.1), snake *E. ocellatus* venom hyaluronidase (ABI33937.1), snake *B.arietans* venom hyaluronidase (ABI33945.1), rat hyaluronidase (AAD01980.1), mouse HYAL2 (AAK28481.1), SFHYA1 (stone fish venom hyaluronidase, AY232496), xenopus kidney hyaluronidase (AAD28281.1), rat sperm surface antigen, 2B1 (CAA62016.1), red fox PH-20 (AAB86588.1), cynomolgus monkey *M.fascicularis* PH-20 (L13780), mouse PH-20 (BAC55070.1), human PH-20 (AAC60607.2), rabbit PH-20 (AAA88913.1) and Guinea pig PH-20 (CAA39768.1).

3.13.5 Expression of BmHYA1 in *E.coli* system

The *E.coli* expression system was employed taking the advantage of its ability to express abundant proteins. In this work, attempts were made to produce recombinant BmHYA1 in *E.coli* expression system using the vector pET41a(+). GST and His tag were applied to facilitate the purification process.

3.13.5.1 Cloning of BmHYA1 cDNA in pET41a(+) vector

pET41a(+) vector combines a strong T7 promoter, which can be recognized by *E.coli* T7 RNA polymerase, and a lac operator repression module allowing the tight regulation of the expression process. The vector also contains multiple cloning sites to facilitate the varying insertion of interest protein genes. The cDNA of BmHYA1 was amplified and structured with restriction sites of *Bam*H I and *Xho* I, or, *Nde* I and *Xho* I, respectively. After double digestion, the BmHYA1 gene was subcloned between *Bam*H I and *Xho* I, or *Nde* I and *Xho* I sites in pET41a(+) vector. After an overnight incubation at 37 ^{o}C, transformants grown on LB plate against kanamycin was randomly selected and those which were confirmed to bear BmHYA1 gene were subcloned into BL21 competent cells. The subcloning resulted in an expression of BmHYA1 fusion protein with a GST or His tag. The structures of the recombinant expression vectors are indicated in Fig.3.34.

(A)

*Bam*HI

TAT CGG GGA TCC *ACA TCT GCC* GAC TTT AAG GTG

Y R G S *T S A* D F K V

*Xho*I

……TGT CAA ATT GCA CCT **TGA** CTC GAG

…… C Q I A P **Stop**

(B)

*Nde*I

CAT ATG CAT CAC CAT CAC CAT CAC *ACA TCT GCC* GAC

H H H H H H *T S A* D

*Xho*I

……TGT CAA ATT GCA CCT **TGA** CTC GAG

…… C Q I A P **Stop**

Fig. 3.34. Nucleotide and amino acid sequences at the region of vector-gene fusion. The nucleotide and the amino acid sequences flanking the vector-BmHYA1 gene are shown. (A) for GST purification, with *Bam*H I and *Xho* I restriction sites. (B) for His purification, with *Nde* I and *Xho* I restriction sites. Restriction sites are underlined. The sequence *ACA TCT GCC* was artificially coded based on Edman degradation result *Threonine-Serine-Alanine*, shown in italics. Stop codon **TGA** is shown in bold type.

3.13.5.2 Expression of recombinant BmHYA1

Transformed BL21 competent cells were incubated on LB plate (with 50 μg/mL kanamycin) overnight at 37 ^{o}C. A single clone was incubated in 5 mL LB-kanamycin medium overnight at 37 ^{o}C with shaking at 220 rpm. Two milliliters out of the previous 5 mL of the inoculated medium was added into 100 mL LB-kanamycin medium (1:50 inoculation). The OD 600 of the medium increased to about 0.5 after 100 minutes with the constant shaking at 220 rpm. The expression of recombinant BmHYA1 was induced

by the addition of IPTG. After 4 hours of IPTG addition, the medium was collected for protein examination. As indicated in Fig. 3.35, a protein with apparent M.W. of 65 kDa was purified by GST tag, which appeared to be more or less in agreement with the M.W. of BmHYA1 (deglycosylated) plus GST fusion protein. The recombinant GST fusion BmHYA1 was soluble, but no obvious activity was detected. In contrast, His-tagged recombinant BmHYA1 was expressed as inclusion body. Apparent protein band at expected M.W. position was detected in whole cell lysates, but no corresponding band was found in supernatant, and nothing was purified from the supernatant (Fig.3.36 (A)). Hence the pellet was denatured to extract the insoluble proteins. Finally, His-tag BmHYA1 was purified from the pellet under denaturing condition. As expected, the M.W. of recombinant His tag BmHYA1 was found to be fairly close to 45 kDa. (Fig.3.36 (B))

3.14 The biological activity investigation of BmHAY1

3.14.1 Direct effect of BmHYA1 on cultured cancer cells

The human breast cancer cell line of MDA-MB-231 is an aggressive cancer cell line that supposedly contains much hyaluronan. Using bHABP (biotinylated HA-binding peptide) as a probe to stain the hyaluronan in the fixed cells, MDA-MB-231 showed abundant quantities of hyaluronan diffused all over the cells (Fig. 3.37 (A)). MDA-MB-231 was treated with BmHYA1 for 20 hours to examine the effect of BmHYA1 on cell hyaluronan breakdown. The enzyme significantly removed the hyaluronan from MDA-MB-231 cancer cells (Fig. 3.37 (B)). Quantitative analysis of DAB staining is shown in Fig 3.37 (C)).

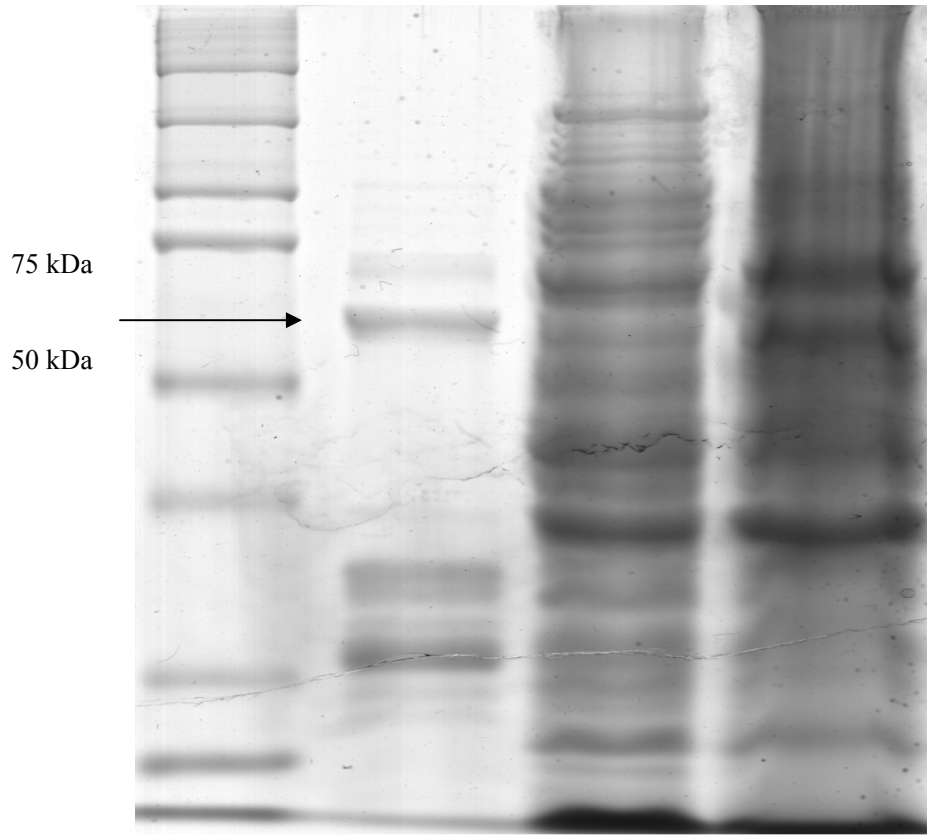

Fig.3.35. Examination of recombinant GST fusion BmHYA1 by SDS-PAGE (12%). Lane1: Protein standards (same as in Fig. 3.1); lane 2: eluate collected from GST affinity chromatography column; lane 3: supernatant of bacterial lysate; lane 4: bacterial whole homogenate. Arrow: GST fusion BmHYA1.

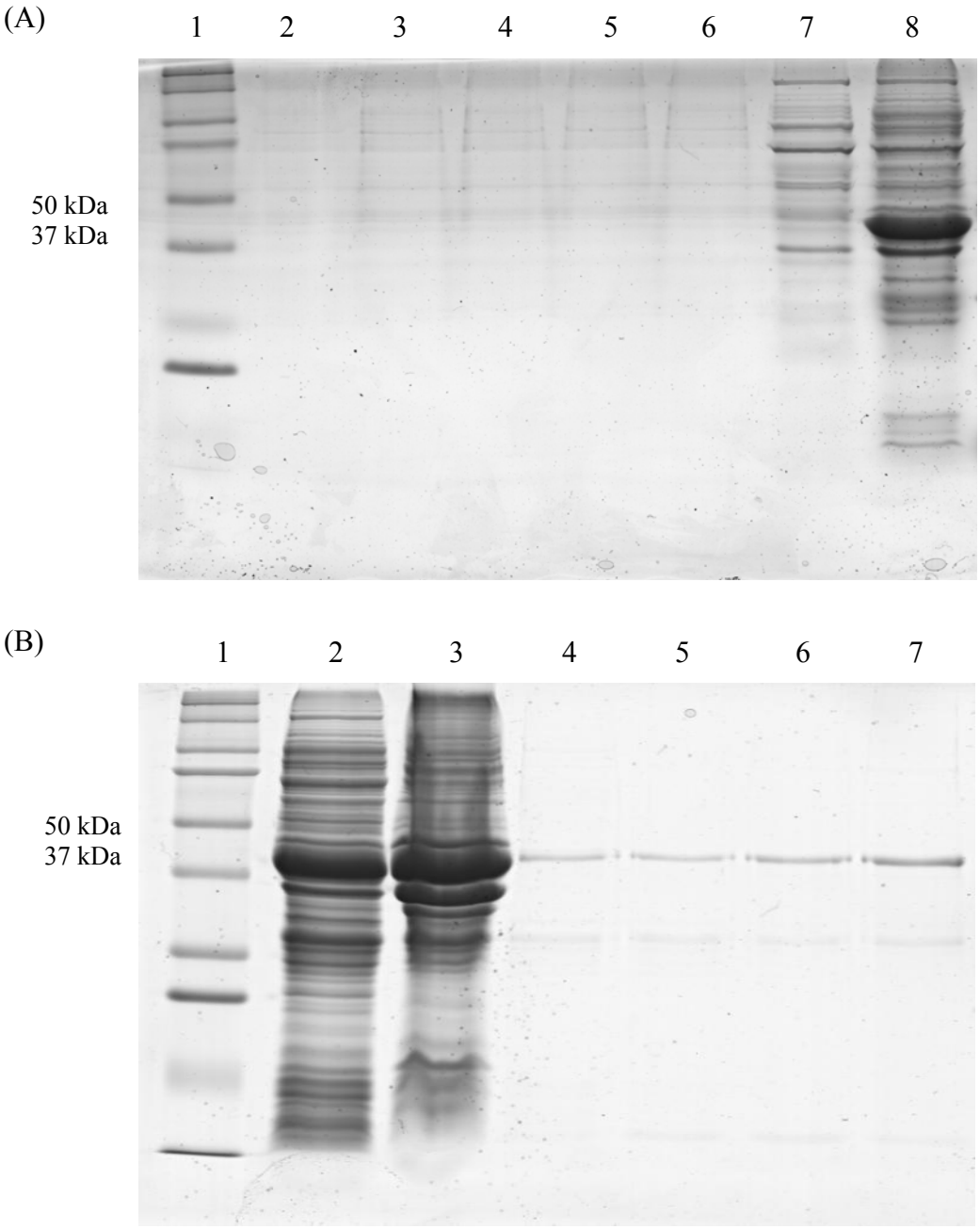

Fig.3.36. Examination of recombinant His-tagged BmHYA1 by SDS-PAGE (12%). (A) Supernatant of bacterial lysate after centrifugation. Lane1: Protein standards (same as in Fig. 3.1); lane 2: blank; lanes 3~6: elution buffer from nickel column (with 10, 20, 75, 250 mM imidazole, respectively); lane 7: bacterial supernatant after centrifuge; lane 8: whole cell lysate. (B) Lane1: protein standards (same as Fig. 3.1); lane 2: whole cell lysate; lane 3: bacterial pellet after centrifugation; lanes 4~7: elution buffer from nickel column (containing 10, 20, 75, 250 mM imidazole, respectively).

3.14.2 BmHYA1 and the expression of CD44 isoforms

To study the effect of environmental hyaluronidase on the expression of CD44 isoforms, western blot was used to examine the expression change of CD44 molecules after the breast cancer cells were incubated with BmHYA1 for different time courses. As shown in Fig.3.38, the expression of CD44v6 was reduced when MDA-MB-231 cancer cells were treated with BmHYA1 for 48 hours while the expression change was not significant when treated for 12 and 24 hours.

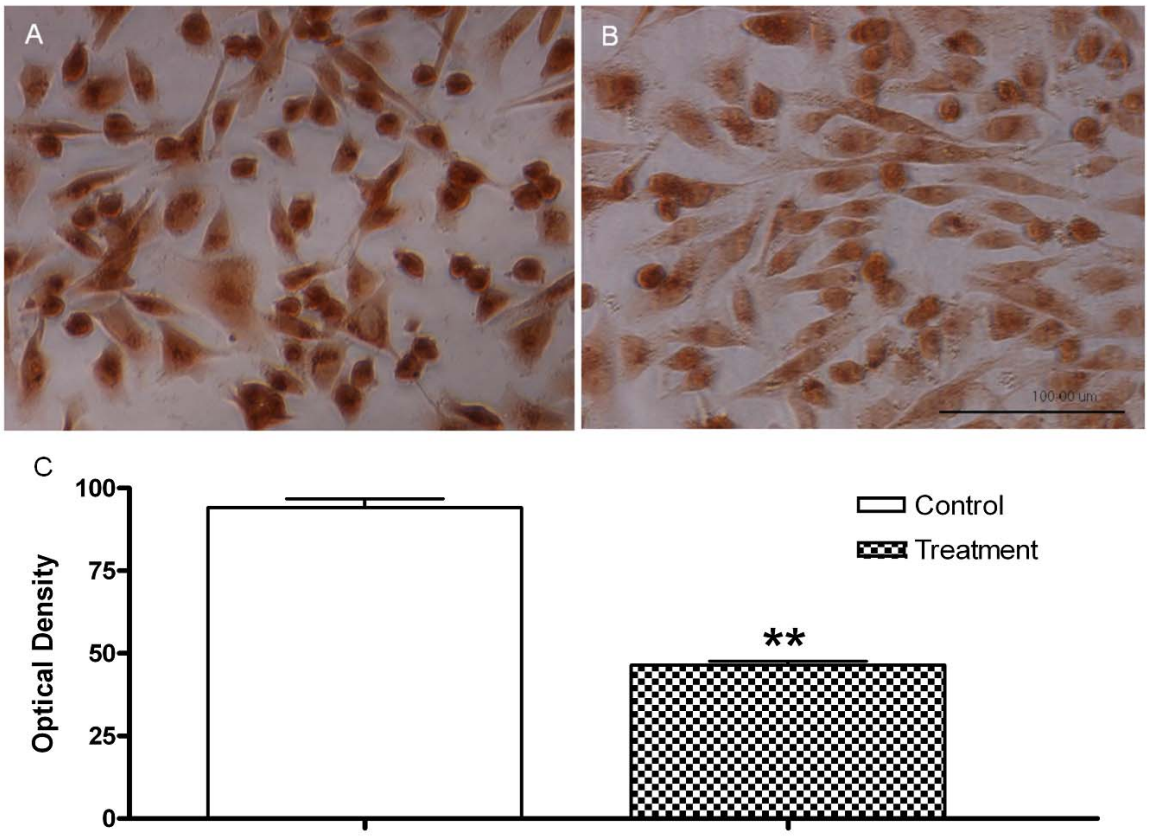

Fig.3.37. Immunohistochemical staining of hyaluronan on MDA-MB-231 cancer cells, with or without treatment with BmHYA1. The cells were incubated at 37 ^{o}C for 20 hours before staining with biotinylated HA-binding peptide (bHABP) overnight at 4 ^{o}C, followed by washing and 1 hour incubation with avidin-labeled horseradish peroxidase solution. (A): Cancer cells without BmHYA1 treatment (control); (B): MDA-MB-231 cells with BmHYA1 treatment. (C): Quantitative analysis of DAB staining for the control (untreated) and BmHYA1-treated MDA-MB-231 cells. Cells (n = 9) were randomly selected from three wells. Vertical bar represents mean ± S.E.M.. **: p<0.01.

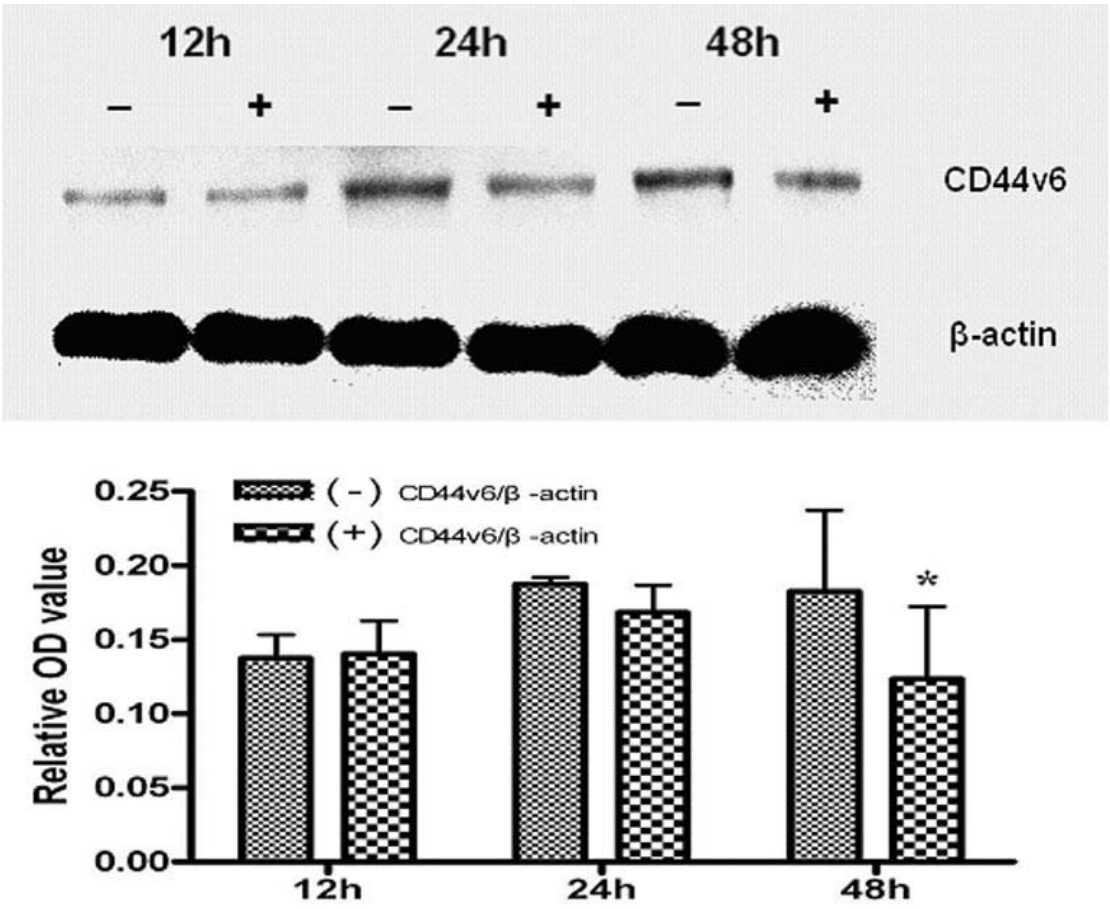

Fig.3.38. The effect of BmHYA1 on CD44v6 expression in MDA-MB-231 cancer cell line. Upper panel: A representative western blot. Lower panel: Quantitation of the immunospecific band for different time points, with and without treatment, respectively. The cells were treated with BmHYA1 for up to 48 hours. There was a significant (*:p<0.05) down-regulation of CD44v6 after 48-hour treatment with 100 units of BmHYA1.

CHAPTER IV

DISCUSSION

4.1 The protein content of crude scorpion venom

The protein content of BCV, as measured by Bio-Rad protein assay, was about 36% of its dry weight. This amount was comparable to 39.04% protein content found in the crude venom of the scorpion *Heterometrus scaber* (Nair and Kurup, 1975). In contrast to scorpion venoms, up to 90% of the dry weight of the crude venoms of snakes was found to be proteins (Mebs, 1969). Bieber (1979) also reported that serine and metalloproteases, PLA_2 and LAAO of snake venoms comprised more than 90% of the dry venom weight. It seems therefore that the protein content of venoms varies between the snake and scorpion species. While the proteins constitute as a principal component in snake venoms, scorpion venoms, on the other hand, contain less proteins. A corresponding increase in non-protein components (e.g., serotonin, histamine, acetylcholine and norepinephrine) might therefore be present in scorpion venoms than that found in snake venoms. The significance of the difference in protein abundance between scorpion and snake venoms might be worthy of further investigation.

4.2 The biological activities of *Bm*K venom

*Bm*K venom has been intensively investigated and even considered as the most studied scorpion venom so far (Goudet et al., 2002). However, the high M.W. proteins of this venom might have not yet been addressed in detail before this study. It is remarkable to note that quite a number of high M.W. proteins, such as serine protease, metalloproteinase, phospholipases and hyaluronidases, have been purified, characterized

and tested from the snake and bee venoms for medical significances (see reviews Markland, 1997; Clemetson et al., 2007; Lee et al., 2008). The high M.W. proteins from the scorpion venom, in spite of the attraction that its low M.W. peptides have received as a source of potential therapeutics, await possible exploitation for drug development. Their characterization should also assist in performing comparative studies between scorpion venoms and those from other species. Hence in screening the biological activity, emphasis was placed on the high M.W. components of this scorpion venom. The BCV was initially separated by the gel-filtration chromatography by virtue of the molecular size difference. The collected peaks from the gel filtration were also examined by SDS-PAGE or MS to estimate the M.W. distribution of the components of each peak. The results clearly show that the high M.W. proteins are concentrated in peak 1 to peak 3, while peak 4 contains a mixture of high and low M.W. fractions, and the later peaks are comprised almost entirely of the low M.W. species. The SDS-PAGE data (Fig. 3.2) obtained with alternate fractions of eluate collected by first 4 peaks indicate that BCV contains many high M.W. proteins (with M.W.>14 kDa). Detection of such high M.W. proteins from the BCV has not been previously reported, and hence BCV can be considered as a rich source searching for new proteins with various biological activities.

LAAO activity

LAAO is known to have a number of biological activities including modulation of the platelet function, induction of apoptosis (Du and Clemetson, 2002), and antibacterial

as well as antiparasitic functions (Ciscotto et al., 2009). As detected during the initial screening procedure, BCV has been found to have only a weak activity. Although LAAO is commonly found in most snake venoms (Du and Clemetson, 2002), it is absent in a number of scorpion venoms including *Buthotus judaicus, Heterometrus spinnifer, Parabuthus transvaalicus, Androctonus australis, \scorpio maurus palmatus, Leiurus quinquestriatus habraeus* and *Pandinus imperator* (Tan and Ponnudurai, 1992). In contrast to those scorpion venoms with devoid LAAO activity, BCV contains a weak LAAO activity that is approximately 70% lower than that found in *Naja kaouthia* snake venom. Although it is weak, the biological activities mentioned above are required to be examined after the purification of the *Bm*K venom LAAO to explore its potential applications. Further study of *Bm*K venom LAAO may also shed light on the evolution of venom LAAOs via comparative studies performed with the snake venom LAAOs.

Fibrinogenolytic activity

The conversion from fibrinogen into fibrin plays an important role in blood coagulation. Fibrinogenolytic proteases may interfere in hemostasis via this conversion process. A number of fibrinogenolytic enzymes have been purified or detected from the venoms of snakes (Shimokawa and Takahashi, 1995; Paes Leme et al., 2008), centipedes (Malta et al., 2008), and spiders (Feitosa et al., 1998; Barbaro et al., 2005). However, no report has been found so far on this activity from scorpion venoms. The results confirm that *Bm*K venom contains fibrinogenolytic activity, and demonstrate that *Bm*K venom

fractions degrade fibrinogen Aα chain, without affecting Bβ and γ chains. Fig. 3.10 (A) shows that the components having the fibrinogenolytic activity are concentrated in the first 3 peaks, indicating the high M.W. (M.W. >14 kDa) nature of these enzymes. The results as shown in Fig. 3.10 (B) and (C) indicate that the fractions from # 55 to 57 contain the components with fibrinogenolytic activity. As compared to other components, fractions # 55 to 57 contain a discrete molecular component with a molecular size between 25 and 37 kDa that is absent in other fractions (Fig. 3.2), and might probably account for the fibrinogenolytic activity of the *Bm*K venom. By substrate preference, the fibrinogenolytic enzymes can be classified into α- or β-fibrinogenase. On the basis of the results obtained so far with *Bm*K venom, it could be assumed that the fibrinogenolytic enzyme(s) of *Bm*K venom belongs to α-fibrinogenase. However, further purification of pertinent fractions followed by time course study is needed to identify and characterize this particular fibrinogenase. In addition, since both serine protease and metalloproteinase may have fibrinogenolytic activity, using PMSF, a specific inhibitor for serine protease, and/or EDTA for metalloproteinase, would allow differentiation of *Bm*K fibrinogenolytic enzyme, according to the specific enzyme type that it belongs to. Again, the fibrinogenolytic activity is rarely reported in scorpion venom, further studies are thus required to explore its properties and potential applications with particular relevance to thrombosis prevention and therapy.

Hemolytic activity

One of the significant effects caused by animal venoms is hemolysis. PLA_2 are probably responsible for hemolysis for many venoms, including snakes (Atanasov et al., 2009; Vogel et al., 1981), honeybee (Watala and Kowalczyk, 1990) and caterpillar (Seibert et al., 2006). Besides PLA_2, some small venom-derived peptides may also account for the hemolytic effect, like Melittin, a 26-amino-acid long peptide from honeybee venom, and some snake cardiotoxins (Fletcher et al., 1996). In this experiment, hemolytic activity was identified in all the 10 chromatographic peaks of *Bm*K venom. The tests were conducted separately for each fraction using (1) whole blood and (2) red blood cells (RBCs) as substrates.

1) On the whole blood, both low and high amounts of BCV showed little effect on the blood cells, whereas all the semi-purified fractions had positive effect, manifesting unambiguous hemolytic activity. This implies presence of various hemolytic factors in *Bm*K venom that could directly affect the blood cells like the high M.W. enzymes (i.e., PLA_2, LAAO, etc.), or low M.W. peptides, such as pore-forming disulfide-bridge-free peptides (Zeng et al., 2005). On the other hand, besides “direct factors”, some other elements like anticomplementary factors might have effect on the hemostatic system and indirectly affect the blood cells. Therefore, the concrete elements present in *Bm*K venom responsible for the whole blood hemolysis are still required the further evidence to confirm.

2) Purified RBCs were used instead of the whole blood to explore the hemolytic factor that could directly affect the RBC. BCV had apparently lower hemolytic effect

than that of the fractions, which might be due to the relatively lower concentration of active components present in it. In the high molecular weight fractions (peak 1~3) the hemolytic effect could be due to the enzymes such as PLA_2 (Jenkins and Olds, 2004), which could directly damage the RBC membrane, while the effect shown by low molecular fractions (peak 4~10) might probably be caused by the peptides with no disulfide bonds, which could form pores in cell membrane with the subsequent release of hemoglobin (Nomura et al., 2004; Nomura et al., 2005). Further studies are needed to elucidate the components which are responsible for the effect.

A very slight hemolytic effect of the crude venom observed on the whole blood and RBC may be due to the presence of relatively low concentration of the effective components in each test sample, or there may be some non-protein protective components in the crude venom. The hemolytic effect of scorpion venom has been studied to a limited extent only. Venkaiah et al. (1983) found the indirect 'complement like' hemolytic effect of the scorpion *Heterometrus fulvipes* venom and stated that it had no direct hemolytic effect on RBC. Bertazzi et al. (2005) reported that *Tityus serrulatus* scorpion venom can activate complement system and indirectly cause hemolysis as well as the inflammatory effect. Up to now, no specific factor that is responsible for the particular effect has yet been identified. Based on the results obtained from this study, it seems possible that both direct and indirect hemolytic factors might probably be present in the *Bm*K scorpion venoms.

Anti-bacterial activity

Using the *Naja Kaouthia* snake venom containing confirmed anti-bacterial activity (Mukherjee, 2007) as a positive control, the *Bm*K venom was found to be devoid of such activity. As showed in Fig. 3.13, BCV and all the 10 fractions showed no antibacterial activity even when the amount of BCV was increased to 1 mg. In contrast, *Naja kaouthia* crude venom showed strong antibacterial activity when used at only one tenth the amount of BCV (i.e., 100 μg). Recently, a novel class of non-disulfide-bridged peptides with unique antimicrobial activity has been successfully cloned from *Bm*K venom gland (Zeng et al., 2005). In view of this, the presence of compounds with antibacterial activity in *Bm*K venom could not be entirely ruled out. It is possible that such antimicrobial peptides may be present in *Bm*K only in trace quantities which could make it impossible for detection because of their low abundance. On the other hand, for high M.W. proteins, since LAAO and PLA_2 enzymes are also known to possess antibacterial activity, it could reasonably be assumed that such enzymatic components in *Bm*K venom might also be present in low or trace amounts only or they have relatively weak antibacterial activity (for this particular bacterial strain DH5α). Nevertheless, other bacterial strains than DH5α are needed to be tested for further antibacterial study of *Bm*K venom.

Amidolytic activity

Amidolytic activity is commonly found in animal venoms. For example, serine protease and metalloproteinase may both have amidolytic activity. Amidolytic activity

was detected in peak 2 of *Bm*K fractions collected after chromatography. Venom amidolytic enzymes were mainly found in snake venoms (Zhu et al., 2005; Salazar et al., 2008). In 2007, He et al. identified a serine protease gene from a sea snake venom gland, and the recombinant protein showed amidolytic activity. Although the enzyme with amidolytic activity was rarely reported for scorpion venoms, the results obtained in this study with *Bm*K venom indicate that this scorpion venom also contains the enzymes with amidolytic activity. Hence, further study is required to characterize the protein, and to identify whether it is a serine protease or metalloproteinase. It would be interesting to compare this enzyme from *Bm*K venom with other venom amidolytic enzymes from other scorpions or other venomous animals.

PLA_2 activity

PLA_2 enzymes are commonly found in most animal venoms. They are secreted from the venom gland hence all are secretory PLA_2s. Venom PLA_2s have various pharmacological effects including neurotoxicity (Strong et al., 1976), cardiotoxicity (Lee et al., 1977), myotoxicity (Ponraj and Gopalakrishnakone, 1995), anticoagulant (Verheij et al., 1980), antiplatelet (Huang et al., 1997) and hemolytic activity (Condrea et al., 1981). PLA_2s are mostly found in snake venoms, and are also present in the venoms of honeybee and lizard (Kini, 1997; Scott et al., 1990; Sosa et al., 1986). As compared to intracellular PLA2s, which have high M.W.s of 31~110 kDa (Mukherjee et al., 1994), the venom PLA_2s have a relatively lower M.W. ranging from 13 to 15 kDa (Kini, 1997).

Despite numerous reports available on snake and bee venoms, reports on scorpion venom PLA2s are hardly found in the literature. Up to now, only one PLA_2 ($IpTx_i$) has been reported from scorpion (*P.imperator*) venom (Zamudio et al., 1997). $IpTx_i$ has a M.W. of about 15 kDa, which is within the acceptable range of M.Ws established for venom PLA_2s. However, the PLA_2 activities identified in this study were located in peak 1 (around fraction #49) and in between peaks 2&3 (around fraction #61). According to M.W. estimations based on the SDS-PAGE size distribution profile, the apparent M.W.s of the two components were found to be more than 100 kDa and around 37 kDa, respectively, which are considered much higher than the commonly M.W. range of $sPLA_2$s reported. On the basis of M.W, it appears likely that the enzyme(s) containing PLA_2 activity in scorpion *Bm*K venom might be classified as a new class of PLA_2. Nevertheless, further purification is necessary to obtain the enzymatically active compound(s) from the fractions to confirm the presence of PLA_2 in *Bm*K venom.

Hyaluronidase activity

Hyaluronidase activity was detected in peak 2, which contained mainly the proteins with M.W. ranging from 19 to 60 kDa, as indicated in SDS-PAGE profile. The M.W. of hyaluronidase obtained from *Bm*K venom is in agreement with those of other scorpion venom hyaluronidases − 47.5 kDa (*Centruroides limpidus limpidus*, Cevallos et al., 1992), 51 kDa (*Tityus serrulatus* venom, Pessini et al., 2001), and 52 kDa (*Palamneus gravimanus* venom, Morey et al., 2006). This hyaluronidase was studied further in detail.

In summary, *Bm*K venom had been found to contain various enzymes besides the abundantly present peptide toxins. In an attempt to look for high M.W. proteins from *Bm*K venom, proteins with various biological activities having M.W.s of over 14 kDa were discovered. *Bm*K venom contains hyaluronidase and LAAO enzymes, and most probably contains serine protease, metalloproteinase and/or PLA_2s, but their presence in the venom could not be confirmed at present.

4.3 Purification and characterization of BmHYA1

BmHYA1 was purified from BCV by 3 stages of chromatography involving gel-filtration, ion-exchange and RP-HPLC. The fact that BmHYA1 was eluted at approximately 70% gradient level of solution B (1 M NaCl and 0.05 M sodium acetate) of cation exchange chromatography indicates that it is a very basic protein like most other venom hyaluronidases. As expected, the theoretical *p*I value of BmHYA1 is later calculated to be 8.65 (see table 1.1). Its M.W, as measured by SDS-PAGE and MALDI-TOF, was found to be 48 kDa and 48,696 Da, respectively. The M.W. estimated from SDS-PAGE is in agreement with that of MALDI-TOF result. Comparison of the M.W.s of BmHYA1 and other scorpion venom hyaluronidases: 47.5 kDa from *Centruroides limpidus limpidus* venom (Cevallos et al., 1992), 51 kDa from *Tityus serrulatus* venom (Pessini et al., 2001), and 52 kDa from *Palamneus gravimanus* venom (Morey et al., 2006) − indicates that BmHYA1 is generally similar in M.W. to other scorpion venom

hyaluronidases. The only exception is the M.W. (82 kDa) of *Heterometrus fulvipus* venom hyaluronidase (Ramanaiah et al., 1990), which is much higher than those of all the other known scorpion venom hyaluronidases, including BmHYA1.

The optimal activity of BmHYA1 (pH 4.5) is similar to that determined for the Indian scorpion (*P. gravimanus*) venom hyaluronidase (Morey et al., 2006), but different from the optimal pH reported for hylauronidases (6.0 and 4.0) from the venoms of *T. serrulatus* (Pessini et al., 2001) and *Heterometrus fulvipus* (Ramanaiah et al.,1990), respectively. It is noteworthy that the optimal pH of all scorpion venom hyaluronidases are all well below 7, and none of such enzymes discovered so far from all animal venoms appear to display the optimal activity at a basic pH (Kemparaju and Girish, 2006; Girish et al., 2002). This indicates that scorpion venom hyaluronidases do not show any apparent activities at pH higher than 7.0.

Interestingly, the optimal temperature (50 ^{o}C) of the scorpion venom BmHYA1 turns out to be more similar to that of the bovine testicular hyaluronidase (Meyer et al., 1960) than to the optimal temperatures of other venom hyaluronidases, including those from scorpion venoms *T. serrulatus* and *P. gravimanus*. The optimal temperatures of those venoms are usually found to be in the relatively lower range of 37~40 ^{o}C (Kemparaju and Girish, 2006; Pessini et al., 2001; Girish et al., 2002; Morey et al., 2006). Surprisingly, it was found that BmHYA1 exerted escalating activity with the rising temperature up to 50

^{o}C. A comparison was made with *Naja kaouthia* venom hyaluronidase and bovine testicular hyaluronidase to confirm this phenomenon. The results showed that the optimal temperature of *Naja kaouthia* venom hyaluronidase was 37 ^{o}C, whereas the bovine testicular hyaluronidase showed the maximal activity at 50 ^{o}C, which was consistent with the report by Meyer et al. (1960). These findings may suggest that BmHYA1 shares some common physicochemical characteristics with mammalian hyaluronidases but differs from those of other venom hyaluronidases. For the effect of temperature on the enzyme, the activity of the enzyme decreased slightly with the gradual increase in temperature from 4~24 ^{o}C, but lost its activity rapidly from 24 °C onwards, indicating it is sensitive to temperature changing at relatively high temperature.

BmHYA1 has the K_m of 95.3 μg/mL, which is not much distinct from the K_m values of the enzymes from *P. gravimanus* (47.61 μg/mL) (Morey et al., 2006) and *T. serrulatus* (69.7 μg/mL) (Pessini et al., 2001). Its catalytic efficiency is much higher than that of stonefish venom hyaluronidase, which has a K_m of 709 μg/mL (Poh et al., 1992), but is much lower than that of *Potamotrygon motoro* fish venom hyaluronidase, whose K_m is 4.91 μg/mL.

BmHYA1 can be inhibited by heparin, Fe^{3+} or Cu^{2+}, but is unaffected by Mg^{2+}, Ca^{2+} or EDTA. Heparin, a non-specific inhibitor to hyaluronidase (Mio and Stern, 2002), is able to inhibit hyaluronidases from the venoms of the snake *Agkistrodon acutus* (Xu et al.,

1982), scorpions *Heterometrus fulvipus* and *Palamneus gravimanus* (Ramanaiah et al., 1990; Morey et al., 2006) and stonefish *Synanceja horrida* (Poh et al., 1992), indicating that heparin has a wide inhibitory spectrum to venom hyaluronidases. The inhibition of heparin to hyaluronidase is non-competitive through formation of a local enzyme complex. As deduced by a Lineweaver-Burke plot, heparin does not bind to the catalytic site of hyaluronidases (Mio and Stern, 2002).

In deglycosylation assay, the shift was observed only with PNGase F treated BmHYA1 (between lane 2 and any lane of 3, 4, or 5 in Fig. 3.27), but not by that treated with either α-2(3,6,8,9)-Neuraminidase or O-Glycosidase (among lanes 3, 4, and 5). According to the product manual, the ability to detect obvious mobility shifts is based on the size of the protein and the relative mass contribution of the sugars removed. If a large protein with a small amount of *O*-linked sugars is treated with O-Glycosidase, it may be difficult to set up the relationship between the presence of *O*-linked sugars and the mobility shifts of the proteins. From Fig. 3.27, the marked shift between lanes 2 and 3 was the result of the removal of *N*-linked sugars by PNGase F, thus indicating the presence of *N*-linked sugars. No obvious shift was detected among lanes 3, 4 and 5, which may suggest that little or no *O*-linked sugar is present in the protein. The overall result indicates that BmHYA1 is a glycoprotein consisting of mainly, if not all, *N*-linked sugars. As one of the most important post-translational modification, glycosylation is commonly found in animal venom proteins. It plays a crucial role in various biological

processes including protein folding, activity exertion and pharmacokinetic stability (Soares and Oliveira, 2009). All the venom hyaluronidases that have been examined so far turn out to be glycoproteins, with the only exception being the hyaluronidase (two isoforms) from the *N. naja* snake venom. Negative result was obtained for *N.naja* venom hyaluronidase with PAS staining test (Girish et al., 2004; Girish and Kemparaju, 2005). The venom hyaluronidases which have not been conducted for glycosylation test may also show the potential *N*-glycosylation sites when their full sequences were deduced and analyzed (Gmachl and Kreil, 1993; Ng et al., 2005; Harrison et al., 2007). The findings of potential *N*-glycosylation sites on their molecules strongly suggest that they would be also glycoproteins, and it might be therefore assumed that most of the venom hyaluronidases are glycoproteins.

4.4 The N-terminal amino acids sequence of BmHYA1

Up to date, amino acid sequences of a number of hyaluronidases from different sources have been identified with sequence homologies noted among them (Kemparaju and Girish, 2006; Stern and Jedrzejas, 2006). The amino acid sequence of the yellow jacket wasp venom hyaluronidase shows 92% and 53% identities respectively, with the hylauronidase sequences of the white faced hornet and honeybee venoms (King et al., 1996). The honeybee venom hyaluronidase exhibits some sequence identity with PH-20, the sperm surface hyaluronidases involved in sperm-egg adhesion, while a number of this PH-20 mammalian sperm protein family members have about 45% sequence identity

with the stonefish venom hyaluronidase SFHYA1 (Gmachl and Kreil, 1993; Ng et al., 2005). For the scorpion venom hyaluronidases, though a few enzymes have been isolated and characterized, the sequence information is rather limited. Before this N-terminal amino acid sequence from the present work, the only partial sequence information (24 N-terminal amino acids) was from the scorpion *T. stigmurus* venom (Batista et al., 2007). Comparison of the N-terminal sequences between BmHYA1 and the hyaluronidase from the venom of scorpion *T. stigmurus* indicates that the two cysteines as well as a lysine are located at the same positions, but no other identical residue is found in the remaining region (Fig. 3.22 (B)). Cysteines in peptides cross link with each other to form disulfide bridge(s), which provide the backbone for peptide folding. The identical cysteine positions of the two scorpion hyaluronidases noted in the present study may suggest the possibility of having a similar backbone to a certain degree in some scorpion venom hyaluronidases. In contrast, some sequence similarity is noted among BmHYA1 and other N-terminal sequences of honeybee and stonefish venom hyaluronidases (Fig. 3.22 (A)). The comparison of thirty N-terminal amino acids of these three venom hyaluronidases indicates that they share identities to a certain degree. BmHYA1 shares approximately 30% of identity to honeybee venom hyaluronidases and 17% of identity to SFHYA1, respectively. The position of first cysteine of BmHAY1 is exactly the same as that found in both of these two proteins. Ng et al. (2005) stated that to get full cDNA sequence of SFHYA1, initial experiment which was based on the previously reported first 20 N-terminal amino acid residues of SFHYA1 (Poh et al., 1992), was unsuccessful and this N-

terminal sequence was later considered to be incorrect. The fact that BmHAY1 has a common feature in N-terminal sequence with other family members suggests the result obtained with Edman degradation is reliable. Further studies are required to solve the complete structure of scorpion venom hyaluronidase in order to understand the function of this enzyme and to elucidate the structure-function relationship.

4.5 The cloning and expression of BmHYA1

The first step of this work was to construct the cDNA library of venom gland. The quality of the library is the cornerstone of the whole following work. In 2001, Zeng et al. reported that for the construction of a *Bm*K venom cDNA library, 60 scorpions had to be sacrificed. Five micrograms of mRNAs obtained from the venom glands of these 60 scorpions were then used for converting to cDNAs, and the ligated cDNAs were finally introduced into *E.coli* cells to complete the construction of cDNA library. In contrast, Schwartz et al. (2007) recently analyzed the transcriptome of the venom gland of a Mexican scorpion *Hadrurus gertschi* using only a single pair of glands. Likewise, only one pair of glands was used for the molecular level study of *Bm*K venom hyaluronidase, suggesting that a pair of venom glands from a single scorpion is enough for the RACE PCR cloning of not-so-rare molecular copies. On the other hand, according to the report by De Lucca et al., (1974), secretion of the secretory epithelium of a snake venom gland was most active after 4 days of milking. As a rule, the RNA extraction from the venom

glands are done following 1~4 days of milking thus allowing the gland epithelium cells to grow to secretory phase for maximal output of the RNA (e.g., Zeng et al., 2001; Schwartz et al., 2007). The present work was accomplished without such pre-treatment applied on the animal. The animal was normally reared and had not been milked before the sacrifice. It indicates that the stimulus of gland epithelium before RNA extraction may not be necessary for a common cloning work.

The present work provides the full-length deduced amino acid sequence of BmHYA1, the hyaluronidase from the venom of *Bm*K. This work presents the first successful molecular characterization of the hyaluronidase from the scorpion venom. The 5' degenerate primer was designed based on the N-terminal sequence. As mentioned before, the lyophilized venom are collected and mixed from a significant number of scorpions (1 gram from 3000 scorpions). Hence the N-terminal sequence information of the protein from the lyophilized venom can be thought of as the representative for this species. Considering that the deduced sequence shares almost the exact N-terminal sequence with the experimental Edman degradation result, it should be also the representative sequence for this particular species. The deduced amino acid sequence together with the N-terminal sequence obtained with the Edman degradation designate the completely mature BmHYA1 protein sequence. The N-terminal sequence of mature BmHYA1 as deduced from the sequence of the cloned cDNA is in agreement with the sequence information obtained by Edman degradation, except for the 23rd position. The

deduced amino acid at 23rd position is an asparagine whereas by Edman degradation there is a cysteine. Since cysteine does not show the signal in Edman degradation chromatogram, it seems possible that some small peaks may be overlooked and hence misinterpreted as cysteine. The deduced result is assumed to be more reliable under the present circumstances.

The mature BmHYA1 comprises 385 amino acid residues. As for the mature protein, the number of amino acid residues of BmHYA1 is comparable to those encoded by some known hyaluronidase cDNAs from other species like human HYAL-1 (384 aa, CAG46731.1), but less than many of other known hyaluronidase enzymes, e.g., *C. elegans* hyaluronidase (436 aa, CAA88874.1) and Guinea pig PH-20 (494 aa, CAA39768.1). BmHYA1 has a theoretical M.W. of 44,549 Da. The difference observed between the theoretical and the experimental (48,696 Da) M.W.s is attributable to the post-translational modification of BmHAY1. Presumably it would be *N*-glycosylations since five potential *N*-glycosylation sites (N-X-S/T) are found in the deduced sequence. These five sites are: N^{23}-V-T, N^{86}-I-S, N^{231}-T-S, N^{360}-P-T, N^{367}-I-S, respectively (see Fig. 3.31). It is notable that the number of potential *N*-glycosylation sites of BmHYA1 is more than the hyaluronidases of honeybee (Gmachl and Kreil, 1993) and SFHYA1 (Ng et al., 2005), both of which contain only 3 sites. The hyaluronidase Eo Hy-1 from snake *Echis ocellatus* venom has also 5 potential *N*-glycosylation sites (Harrison et al., 2007). Their contribution to the M.W. of the native enzymes needs to be determined. It is also notable

that none of the five potential *N*-glycosylation sites in BmHYA1 sequence represents in other hyaluronidase sequences. Meanwhile, BmHYA1 also lacks of the highly conserved potential *N*-glycosylation site of hyaluronidases (positions 323-325 of BmHYA1 sequence, see Fig. 3.32). It may suggest that BmHYA1 possess certain special structure, compared with other hyaluronidases.

BmHYA1 has 12 cysteines and these cysteines might have different suggestions (Fig. 3.32). It has been elucidated that the two disulfide bonds of honeybee venom hyaluronidase are formed by Cys^{22}-Cys^{313} and Cys^{189}-Cys^{201} (Marković-Housley et al., 2000). In BmHYA1 sequence, it could be seen that the conservation of cysteine residues at Cys^{16} and Cys^{306} as well as Cys^{181} and Cys^{195} indicates this scorpion venom hyaluronidase has probably the same partial structural feature as honeybee venom hyaluronidase. Similarly, the conservation of Cys^{331}, Cys^{336}, Cys^{342}, Cys^{370}, Cys^{372} and Cys^{381} between BmHYA1 and hyaluronidases other than honeybee venom hyaluronidase, may also suggest the structural significance of BmHYA1, though it remains as a conjecture since no experimental evidence is available at this point so far. Still, we notice that Cys^{174} and Cys^{217} are not consensus at all with any other hyaluronidases. It might be assumed that these two cysteines form special disulfide bond of BmHYA1 and hence likely lead to its special structural property.

In 2007, Batista et al. reported the first partial amino acid sequence of the scorpion (*T.*

stigmurus) venom hyaluronidase. BmHYA1 was initially found to have two cysteine residues in N-terminal sequence identical to those of this *T.stigmurus* scorpion venom hyaluronidase, hence it was assumed that these cysteines may form special structure for scorpion venom hyaluronidases (Feng et al., 2008). Further investigation revealed that the sequence of this *T. stigmurus* scorpion venom 'hyaluronidase' may not belong to hyaluronidase family. It is almost identical to the N-terminal amino acid sequence of an allergic protein from Brazilian yellow scorpion (*Tityus serrulatus*) venom (Swiss-Prot accession no. P85840.1), and also has higher similarity to N-terminal sequences of other allergic proteins (e.g., Swiss-Prot accession no. P85860.1, GenBank accession no. ABX75373.1, both from spider venoms) than those of hyaluronidases. Hence it is highly unlikely that the protein could be hyaluronidase. On the other hand, as mentioned above, from the deduced BmHYA1 sequence, it was found that the 23rd position of BmHYA1 is not a cysteine, but an asparagine. The former was experimentally obtained by Edman degradation. This discrepancy may be due to the misreading of Edman degradation chromatogram. Since cysteine showed no signal in the graph, it seemed likely that some weak peak may escape detection and hence recognized as cysteine by mistake. If it is true, the total number of cysteines is 12, which is in agreement with the usual status of even number of cysteines, to form the disulfide bonds. Therefore it is assumed that the second cysteine, which is located at the 7th position downstream to the first cysteine in BmHYA1 N-terminal sequence, may not exist. However, a new hyaluronidase N-terminal sequence (34 amino acids, Swiss-Prot accession no. P85841.1) from the venom of Brazilian yellow

scorpion (*Tityus serrulatus*) demonstrates that the second cysteine appears at the 7th position downstream to the first cysteine and also has high identity with the N-terminal sequence of BmHYA1 (Fig. 4. 1). If it is the case that the 23rd residue in BmHYA1 protein sequence is really a cysteine, but not an asparagine, it may strongly suggest that there should be still a special structure in scorpion venom hyaluronidases. Nevertheless, further confirmation awaits the discovery of more new scorpion venom hyaluronidase sequences.

```
          10         20         30           Source
TSADFKVVWE VPSIMCSKKF KICVTDLLTS HKILVNQ    (A)
   KFKVYWE VPSFLCSKRF KICVTEVLTS HEILVNQ    (B)
```

Fig .4.1. The comparison of N-terminal sequences of (A) BmHYA1 (Feng et al., 2008; present work) and (B) Brazilian yellow scorpion (*Tityus serrulatus*) venom hyaluronidase (Swiss-Prot accession no. P85841.1). The two sequences are highly identical and both have the 6-amino-acid-residue interval between the two cysteines. The identical residues are shaded and the cysteines are highlighted in white color. The 23rd amino acid residue (the second cysteine position, underlined) in BmHYA1 sequence is asparagine as deduced by nucleotide sequence.

The other structural characteristic of interest of BmHYA1 is the determinants for its catalytic action. Based on the three-dimensional structure analysis of honeybee venom and bovine PH-20 hyaluronidases, a cleft in both proteins is a potential catalytic site, which contains a catalytic Glu and a number of positioning residues. The mutation of involved residues may lead to the alteration of enzymatic behaviors (Stern and Jedrzejas,

2005). In the deduced BmHYA1 protein sequence, the corresponding elements are also identified. The catalytic Glu is located at the 105^{th} position of BmHYA1, and the surrounding positioning residues are supposed to be Asp^{103}, Tyr^{176}, Tyr^{221} and Trp^{294}, which are exactly the residues located at the equivalent positions of the tertiary structures of honeybee venom and bovine PH-20 hyaluronidases (Fig. 3.22). It has been suggested that the substrate specificity difference of Human HYL-4 is attributable to mutation in the positioning residues of Tyr to Cys (263^{rd} position) in Human HYL-4 (Stern and Jedrzejas, 2005. Note: Cys^{263} has been updated to be Gly^{263} in sequence version 2). Hence it seems likely that BmHYA1 might have the same substrate specificity as that of honeybee venom hyaluronidase and bovine PH-20.

The expression of BmHYA1 was carried out in *E.coli* expression system, which is the method of choice for heterologous expression of recombinant proteins due to its capacity to produce abundant recombinant proteins and easy manipulation. Enzymatically active recombinant venom hyaluronidase has been successfully expressed in *E.coli* system (Gmachl and Kreil, 1993). In that work, the recombinant bee venom hyaluronidase was purified from the inclusion bodies. After reduction and refolding, the recombinant protein demonstrated about 80% of the native hyaluronidase activity. However, several failed trials had also been reported in heterologous expression of hyaluronidase in *E.coli* system. Gmachl et al., (1993) reported that recombinant human PH-20 hyaluronidase expressed from *E.coli* failed to show any detectable activity. Ng. et al. (2005) successfully cloned stonefish hyaluronidase SFHYA1 cDNA and expressed it

in insect cells in its active form, but not in *E.coli*. In a more detailed expression study, Soldatova et al. (1998) compared the bee venom hyaluronidase expression in prokaryotic and eukaryotic systems, and revealed that the protein expressed in insect system had the activity comparable to the native protein, while the protein expressed in *E.coli* formed inclusion body with the reconstituted protein showing only 20~30% of the activity of the native bee venom hyaluronidase. This indicates that the post-translational modifications, such as glycosylation, may play a key role for correct conformation of the hyaluronidases. In the present work, *E.coli* expression system was employed, along with the two purification tags, i.e., GST and His. GST improves the solubility of the recombinant protein and at the same time providing a tag for protein purification, hence it is commonly used as an expression tag in recombinant protein production. The result obtained from the GST tag expression and purification showed that the expression level was not satisfactory, and the protein turned out to have no enzymatic activity. Since the *E.coli* system is not efficient enough to provide appropriate post-translational modification, it is likely that the protein may fail to fold properly. Another possibility is that the size of BmHYA1 is not much bigger than that of GST, and this may influence the biological activity of recombinant BmHYA1. To counteract this effect, His tag was employed as an alternative purification strategy. Although His tag is relatively smaller in size containing only 6 Histidine residues, it may cause aggregation of the heterologous protein in the host cells, and that was what happened in the present work. The protein was insoluble and additional reducing and refolding process should be taken to reconstitute

the protein for further study. Otherwise the eukaryotic expression system, such as insect cells, may be introduced.

4.6 Biological activities of BmHYA1

Hyaluronidase can degrade hyaluronan to a wide range of fragments in different sizes. Human HYAL-2 degrades hyaluronan to 20 kDa products (Lepperdinger et al., 2001) while Human HYAL-1 hydrolyzes the substrate into tetrasaccharides (Csoka et al., 2001). Among the five hyaluronidases of human origin, only these two are well characterized. Further related studies have been done for the hyaluronidases from venoms. Sugahara et al. (1992) studied the reaction product of the stonefish venom hyaluronidase and found the products to be mainly tetra-, hexa-, octa- and deca-saccharides, while no disaccharides were found. The hyaluronidase from *Agkistrodon contortrix contortrix* (snake) venom hydrolyzed the substrate into hexa- and tetrasaccharides (Kudo and Tu, 2001), while the final products were tetrasaccharides for *Naja naja* (snake) venom hyaluronidase (Girish et al., 2004). BmHYA1 was supposed to be the first scorpion venom hyaluronidase whose final degradation production was analyzed. It was found to hydrolyze the hyaluronan into relatively small size oligosaccharides of tetrasaccharides (the degradation products of hyaluronan by venom hyaluronidases were summarized in table 4.1). Since low M.W. hyaluronan fragments are known to play a role in inflammation, immunostimulation and angiogenesis (Stern, 2005), it is possible that the venom hyaluronidases, besides acting as the spreading factor, may also contribute to local

tissue damage and inflammation through the action of hyaluronan fragments on connective tissue, collagen and blood vessels.

Table 4.1. Comparison of the degradation products by different hyaluronidases

Source	Products	Reference
*Bm*K, Scorpion	tetrasaccharides	Present work
S. horrida, Stonefish	tetra-, hexa-, octa- and deca-saccharides	Sugahara et al.,1992
A. contortrix contortrix, Snake	hexa- and tetrasaccharides	Kudo and Tu, 2001
N. naja, Snake	tetrasaccharides	Girish et al., 2004

Hyaluronan has important physiological as well as pathological functions. It is up-regulated in breast cancer, with abundant levels of hyaluronan often found to be correlated with tumor aggressiveness (Götte and Yip, 2006; Anttila et al., 2000). Hyaluronan also plays a special role in cancer cell invasion and metastasis by interacting with CD44, a member of a big family of cell transmembrane glycoproteins involved in cell-cell and cell-matrix connections (Delpech et al., 1997). Cancer cells express high levels of CD44. Variants of CD44 (CD44v) are generated by alternative splicing of a single gene (Naor et al., 1997). Being the primary receptors of hyaluronan, they are able to respond to environmental hyaluronidase signals. Following 20 hours incubation of

cancer cell line MDA-MB-231 with bovine testicular hyaluronidase, CD44v6 mRNA was almost eliminated, with CD44v6 protein no longer detectable by immunohistochemical staining. This effect has been suggested to occur as an indirect way of eliminating the network-like presence of hyaluronan on the cell membrane (Stern et al., 2001). Treatment with bacterial hyaluronidase also resulted in the suppression of CD44 expression at tissue level (Laugier et al., 2000). Contrary to these findings, CD44 and CD44v mRNAs were reported to be up-regulated after 12-hour treatment with bovine testicular hyaluronidase in rabbit articular chondrocytes (Ohno-Nakahara et al., 2004), suggesting that the mechanisms for the CD44 modulation between human breast cancer cell and rabbit articular chondrocytes may be different. Our findings showed that for breast cancer cell line MDA-MB-231, hyaluronan was considerably removed from the cells after incubation with the hyaluronidase BmHYA1 for 20 hours. Based on the protein levels, it is suggested that the CD44v6 is down-regulated after treatment of the human breast cancer cell line MDA-MB-231 with BmHYA1 for 48 hours. This confirms that the CD44 variant of cancer cells can be modulated by external signaling by way of external hyaluronidase treatment. The mechanism for such signaling events will be further investigated in relation to treating resistant cancers, which may also lead to identifying novel pathways for the regulation of CD44 and its variants.

4.7 Future directions

1) To continue to examine some other candidate proteins in *Bm*K venom which were not

involved in the present screening, such as nerve growth factor and C-type lectin-like protein, and study further the identified enzymes/activities, such as LAAO, amidolysis and fibrinogenolysis.

2) To conduct further characterization study on substrate specificity. By combining the information obtained from sequence alignment and phylogenetic tree of hyaluronidases, the evolutionary process of hyaluronidase enzyme could be well assumed.

3) To obtain the full-length BmHYA1 cDNA by exploring further its 5' end nucleotide sequence using RACE technique.

4) To produce active recombinant BmHYA1 protein. The protein expressed by His purification tag can be refolded in order to get the activity recovered. Alternatively, the protein can be expressed by eukaryotic expression system, like insect cells Sf9. Furthermore, the mutagenesis studies can be carried out to investigate the structure-function relationship.

5) To study BmHYA1's crystal structure. With abundant recombinant active BmHYA1, it is possible to grow the crystal and subsequently study its secondary/tertiary structure by X-ray crystallography.

6) To study BmHYA1's medical application. RNA level, tissue level and animal level experiments may be applied to further study the effect of hyaluronidase on CD44 molecular regulation, pertaining to breast or prostate cancer biology.

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